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ACUTE ANOXIA AND THE EXCRETION OF WATER AND ELECTROLYTE

BY ELISABETH ULLMANN

From the Department of Physiology, The Medical College of St Bartholomew's Hospital, London, E.C. 1

(*Received* 12 *May* 1960)

If men are exposed to acute anoxic anoxia a variety of alterations in water and electrolyte excretion may occur. Diuresis has been noticed by several observers who studied the physiological effects of real or simulated high altitude (McFarland & Edwards, 1937; Burrill, Freeman & Ivy, 1945; Asmussen & Nielsen, 1945; Schönholzer, Gross & Marthaler, 1948). 'Höhendiurese', familiar to mountaineers, was found by Stämpfli & Eberle (1944) to continue at 3400 m for several days, but only if the altitude was well tolerated. Victims of mountain sickness became oliguric on the day after arrival. Ochwadt (1947) observed that at a simulated altitude of 6000 m water diuresis was enhanced, but if the subjects had not been given a large waterload they became oliguric. McCance (1935) described long-lasting oliguria and profound depression of urea and electrolyte output following severe anoxaemia. Most other workers have found solute excretion—notably of sodium, potassium and chloride—to rise during anoxia, irrespective of changes in the rate of urine flow (Stämpfli & Eberle, 1944; Burrill *et al.* 1945; Asmussen & Nielsen, 1945; Schönholzer *et al.* 1948; Fishman, Maxwell, Crowder & Morales, 1951; Axelrod & Pitts, 1952). Berger, Galdston & Horwitz (1949) found no consistent changes in potassium and water elimination, but came to regard the 'invariable and frequently marked increase in the rate of sodium and chloride excretion' as the characteristic renal effect of anoxic anoxia.

Clearance studies have shown that the renal circulation usually remains unaltered when human subjects breathe gas mixtures containing about 10% oxygen. If a change occurs it is an increase, not a decrease of renal plasma flow, with or without a slight rise of filtration rate (Berger *et al.* 1949; Fishman *et al.* 1951; Axelrod & Pitts, 1952).

Acute anoxic anoxia, besides causing cardio-vascular changes also leads to hyperpnoea and respiratory alkalosis, and to alterations in the activity of some of the endocrine glands. It was the purpose of the present work to investigate whether the many different modifications of renal function

which have at various times been observed in anoxia are directly related to particular extra-renal effects of systemic oxygen lack.

METHODS

Subjects. The subjects were healthy adults of both sexes, aged 19–44 years. With few exceptions they were ignorant of the nature and purpose of the experiments. Their activity, diet and fluid intake were not controlled between experimental days. Tea or coffee, but not smoking, were prohibited during all experiments and the preceding 12 hr.

Procedure. Most experiments were conducted during the morning. So as not to mistake changes governed by the diurnal excretory rhythm for experimental results all tests consisted of two or more separate sets of observations with the same subject, carried out at

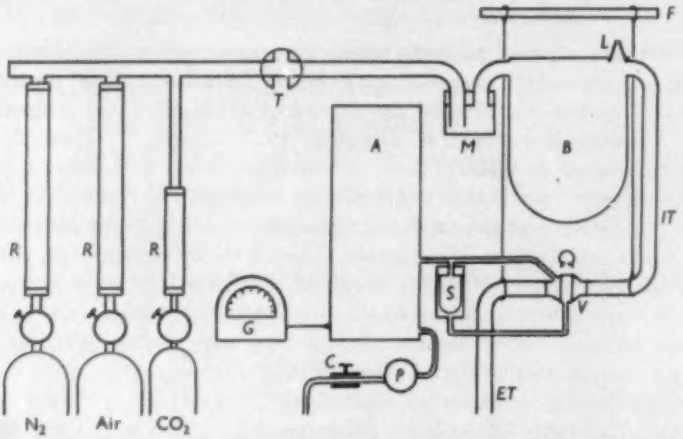

Fig. 1. Administration of gas mixtures and recording of end-expiratory CO_2 concentration. *A*, 'infra-red' CO_2 analyser; *B*, meteorological balloon serving as reservoir; *C*, clamp regulating flow through analyser; *ET*, expiratory tubing; *F*, frame; *G*, recording galvanometer; *IT*, inspiratory tubing; *L*, leak; *M*, mixing and moistening chamber; *P*, suction pump; *R*, rotameter; *S*, Rahn–Otis sampler; *T*, 3-way tap; *V*, modified Siebe–Gorman valve. For further details see text.

exactly the same time of day on different days. One of these was a control run. The sequence of 'control' and 'experimental' runs was randomized. Intervals between the several runs which constituted a complete test on one subject varied from 2 days to 3 weeks; most tests were completed inside 10 days. Further details are given in the text.

Administration of gas mixtures and continuous recording of end-expiratory CO_2 concentration. The subject wore a nose clip and breathed through a modified standard Siebe–Gorman valve with identical centrally fixed rubber disk valves on the inspiratory and expiratory sides. The dead space of the valve was reduced to 36 ml. Except in the first series of experiments oxygen-poor gas mixtures were always administered by means of the apparatus illustrated in Fig. 1. This was similar in concept to that described by Cunningham, Cormack, O'Riordan, Jukes & Lloyd (1957) but differed from it in many details.

Nitrogen, air (or oxygen) and, if used, carbon dioxide were led from upright cylinders

through three separate, suitably calibrated rotameters (*R*) into a joint inlet tube, from there through wide-bore (diameter 2·54 cm) flexible tubing to a moistening and mixing chamber, and hence to a reservoir (*B*). A wide-bore three-way tap (*T*) was inserted into the tubing. At the start of work this tap stood open to the room until the rotameters were all set at the flow rates calculated to give the desired gas mixture. Delicate adjustment of the flow rate through the rotameters was achieved by means of two-stage reducing valves ('Endurance' pressure-reducing regulator, B.O.C. ref. No. MS 51) on the gas cylinders. When the rotameters were steady, tap (*T*) was turned and from then on a gas mixture of known composition flowed into the reservoir (*B*).

The reservoir was a thin rubber bag made from a meteorological balloon with separate inlet and outlet channels. It held approximately 30 l. when distended but offered practically no resistance to inflation until the contained volume exceeded 12 l. Its outlet channel led into the straight part of a wide metal T-piece, the side arm of which carried a rubber non-return valve acting as a leak (*L*). Distal to this an adjustable slit across the lumen of the straight tube controlled the flow of gas from the reservoir through a further length of flexible wide-bore tubing (*IT*) towards the inspiratory valve. The resistances of the slit and of the leak were so balanced that, if the inflow from the rotameters exceeded the subject's ventilation rate and the pressure in the reservoir began to rise, gas escaped through the leak and none would push through the inspiratory valve (*V*) under its own pressure unless aspirated. The expired air was collected in a further 1·22 m length of flexible tubing (*ET*) pointing downward to allow drainage of saliva. The valve and tubing were suspended from a rigid iron frame (*F*). The subject sat in a comfortable chair and could breathe through the mouthpiece without altering his posture or straining any muscles. The composition of the gas mixture breathed was checked at intervals with a Haldane gas analysis apparatus.

Alveolar air—or rather 'end-expiratory' air—was sampled continuously by the method of Rahn & Otis (1949) and passed through an 'infra-red' CO_2 analyser (*A*) (Infra-red Development Co., Welwyn Garden City, Herts; Model SC/M, modified). The flow of end-expiratory air through the analyser was varied between 60 ml. and 200 ml./min, depending on the rate of breathing. Immediately before recording began, and immediately after it ended, the analyser was standardized with two or three mixtures of CO_2 in oxygen, the exact composition of which had previously been determined with a Haldane apparatus. Over the range of concentrations found in the experiments the calibration points lay on a straight line, and changes of concentration of 0·05% over a range from 0 to 7% CO_2 could be measured. Records of CO_2 concentration were made every 30 or 60 sec during tests.

'Alveolar' CO_2 concentration could be maintained constant, if desired, by varying the CO_2 concentration of the inspired gas mixture according to the observed trend of the end-expiratory CO_2 concentration. It was found that when the reservoir (*B*) contained less than 6 l. of gas mixture a fall in end-expiratory CO_2 concentration could be reversed and the control value restored in less than 1–1½ min. Spontaneously arising increases in end-expiratory CO_2 concentration could, of course, not be corrected by this method but these occurred rarely and were always short-lived.

Blood and urine specimens. Blood samples were drawn anaerobically from the antecubital vein. Urine specimens were obtained by voluntary micturition. Subjects unable to empty the bladder completely, or at specified times, were not used. Specimens were passed into narrow graduated cylinders containing a 5 cm layer of paraffin, and left to attain room temperature.

Chemical analyses. The pH of blood and urine was measured with a miniature glass electrode in special containers at room temperature. Readings were corrected to body temperature according to the procedure of Rosenthal (1948). CO_2 and O_2 content of whole blood, and CO_2 content of plasma and urine were determined manometrically (Peters & Van Slyke, 1932). Bicarbonate concentration of blood and urine was calculated by the Henderson–Hasselbach equation.

Other substances were estimated as follows:

Endogenous creatinine in serum: Delroy (1949), and King & Wootton (1956). When the methods were compared using dogs' serum the latter gave values 25–30% lower than the former. With neither method could changes in serum creatinine concentration *during a single experiment* be detected. Colours were compared in a spectrophotometer (Unicam SP600). Endogenous creatine in urine: Delroy (1949); colours were compared in an EEL colorimeter.

Urea in blood, serum and urine, and ammonia in urine: by the micro-diffusion technique of Conway (1950).

Plasma or serum protein: Phillips, Van Slyke, Hamilton, Dole, Emerson & Archibald (1950).

Glucose and protein in urine: qualitative tests for the presence of traces were performed according to Cole (1941).

Sodium and potassium in serum and urine were estimated with an EEL flame photometer.

Chloride in serum or plasma: Schales & Schales (1941). Chloride in urine: modified Volhard-Harvey titration (Peters & Van Slyke, 1932).

Inorganic phosphate in serum and urine: King & Wootton (1956).

The concentration of buffered hydrogen ions in urine was calculated from the inorganic phosphate content and pH of the urine. Urine osmolarity was calculated from the depression of the freezing point, determined with a Beckmann thermometer. Readings accurate to 0·002° C could be taken (± 2·5 m-osmole/l.).

RESULTS

Comparison of anoxia accompanied by hypocapnia with simple hypocapnia

Systemic oxygen lack induces hyperpnoea and respiratory alkalosis. It was therefore necessary to examine first whether and in what respects the apparent renal effects of anoxia differed from those of simple hypocapnia.

Six subjects took part in two series of tests. In one they were moderately dehydrated, having had no food or fluid for 18 hr, and they drank no water during the experiment. In the second series the same subjects were in a normal state of hydration. They had a standard breakfast 2 hr before the start of observations; at the end of an initial control period lasting 60 min they drank a volume of tap water which differed from subject to subject (350–750 ml.), but was the same on all occasions for any one person.

Observations extended over 3 hr on all occasions. Experimental procedures, if any, were carried out during the second hour. The subject either overventilated his lungs deliberately with room air, or breathed a gas mixture of constant composition from Douglas bags. Various mixtures employed contained between 14 and 10% oxygen in nitrogen. Urine specimens were collected at the end of the first hour and at 30 min intervals thereafter. Three subjects were unable to empty their bladders at the specified time when the rates of urine flow were very low; because of this only three control runs could be successfully completed in the 'dehydrated' group. Blood samples were taken just before, and at the end of experimental procedures, but not from all subjects.

Solute excretion. During inhalation of 10–14% oxygen in nitrogen the excretion of potassium, sodium, bicarbonate and chloride rose, ammonium excretion diminished and urinary hydrogen ion concentration fell in all subjects, as shown by the circles in Fig. 2. When the same subjects overventilated their lungs with room air (triangles in Fig. 2), very similar

changes occurred. They are typical of respiratory alkalosis (Stanbury & Thomson, 1952; Barker, Singer, Elkinton & Clark, 1957). Both procedures increased sodium and chloride excretion to the same extent, but the other changes were smaller in anoxia, probably because anoxia caused a smaller

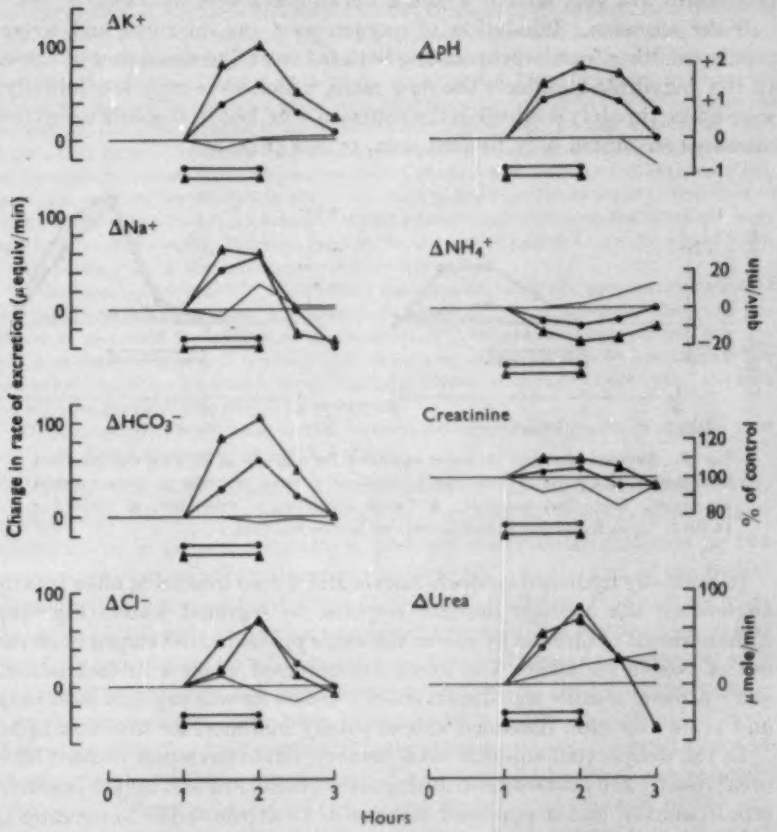

Fig. 2. Average changes in urinary solute excretion in a group of six dehydrated subjects during forced overbreathing of room air (▲), and during inhalation of 14–10% oxygen in nitrogen (●) in the period indicated by horizontal bars. Thin line, controls. In this and in other figures the ordinates show the average change from the rates of excretion in the initial control period.

loss of CO_2 from the blood than voluntary overbreathing (Table 1). Differences between the responses of individuals were merely of degree. Figure 2 shows the average changes found when the subjects were dehydrated; when the subjects were well hydrated during the experiment

the changes in creatinine and electrolyte excretion were of the same order of magnitude. The rate of urea excretion in hydrated subjects was much higher both in the control runs and during experimental procedures, but the actual increment in urea output attributable to the experimental procedures was very similar whether the subjects were hydrated or not.

Water excretion. Inhalation of oxygen-poor gas mixtures and hypocapnia resulting from overbreathing both led to an increased flow of urine. In the dehydrated subjects the flow rates, which were very low initially, were approximately doubled in the course of 1 hr, but in absolute terms the increases amounted only to 1 ml./min, or less (Fig. 3*a*).

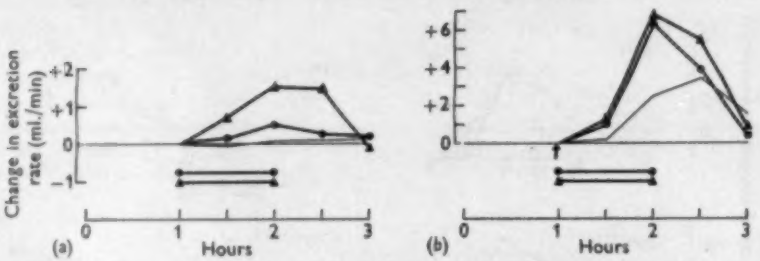

Fig. 3. Average changes in water excretion for a group of six subjects (*a*) when dehydrated, and (*b*) after drinking a standard volume of water at time marked by arrow. Thin line, controls. ▲ forced breathing of room air; ● breathing 14–10 % O_2 in N_2 during period shown by horizontal bars.

In normally hydrated subjects anoxia and forced breathing alike greatly augmented the average diuretic response to ingested water (Fig. 3*b*). Enhancement of diuresis by one or the other procedure developed in eleven out of twelve instances. The exception occurred when a subject fainted while a blood sample was drawn shortly before he was exposed to anoxia, and urine excretion remained almost totally inhibited for the next 2½ hr.

In the dehydrated subjects total urinary solute excretion [2(Na + K) + urea] rose by 390 μosmole/min during forced breathing and by 310 μosmole/min in anoxia, and it appeared reasonable to attribute the lower rates of urine flow found in anoxia (Fig. 3*a*) to the lower osmotic activity of the urine. In the hydrated subjects the maximum increase of urinary solute excretion during forced breathing and anoxia was 575 and 290 μosmole/min, respectively, yet the difference was not paralleled by a difference in the volume of the urine (Fig. 3*b*). The high average urine flows found in anoxia were all the more surprising as the mean included one instance of complete suppression of the diuretic response to water after fainting. These results suggested that systemic oxygen lack might enhance diuresis by some mechanism unconnected with respiratory alkalosis and raised solute

excretion. A method of inducing anoxia without concomitant hypocapnia was therefore devised.

Comparison of anoxia with and without accompanying hypocapnia

The six subjects of this series of tests had not taken part in earlier experiments. They were normally hydrated, and had a breakfast of fixed composition 2½ hr before the start of observation. Observations were made during 4 hr which were subdivided into six urine collection periods (1–6) lasting 60, 30, 30, 30, 30 and 60 min, respectively. All subjects, whatever their body size, drank 500 ml. of tap water at the end of period 1 on all occasions. They were seated throughout the period of observation, and stood up only to pass urine. During the control runs they breathed room air without valve or nose clip. If they were to be made anoxic they began, 30 min after the start of period 1, to breathe room air through the valve of the apparatus described in the section on 'Methods'. Their resting end-expiratory CO_2 level was recorded for 25–30 min before the start of the experiment proper. Exposure to anoxia began with period 2, immediately after the subject had passed the first urine specimen and had drunk the water. It lasted for 1 hr, to the end of period 3, save for a brief interruption during which the second urine sample was voided.

In experiments without CO_2 replacement the composition of the gas mixture breathed was kept constant throughout, and consisted of 11% oxygen in nitrogen. When CO_2 loss was to be prevented the basic mixture contained 10% oxygen in nitrogen and to this was added as much CO_2 as was required from minute to minute to keep end-expiratory CO_2 concentrations at the pre-anoxic level. Since the volume of the admixture varied the final oxygen concentration also fluctuated somewhat.

When the subjects were made anoxic, but not during control runs, blood samples were taken just before the close of periods 1, 3 and 6.

Systemic effects of anoxia. After a transient initial disturbance which varied from subject to subject the effect of anoxia on pulse rate and blood pressure was in general very slight, and not consistently modified by the presence or absence of simultaneous hypocapnia. At the end of the period of anoxia it was frequently difficult to draw an adequate blood sample from the antecubital vein owing to vasoconstriction in the extremities manifested by intense pallor and coldness to touch. These signs were equally prominent whether or not anoxia was accompanied by hypocapnia, contrary to what might have been expected from Black & Roddie's (1958) observations during brief periods of very intense anoxia. In both types of experiment the subjects complained of feeling cold, of mild or severe headache, and of increasing drowsiness. Disorientation at the end of the exposure was usually more pronounced in anoxia with hypocapnia. Several subjects were unable to empty the bladder at will during or immediately after anoxia while breathing air. This difficulty was not related to hypocapnia. Impaired bladder control in anoxia has previously been noticed by Ochwadt (1947) and by Axelrod & Pitts (1952).

Two subjects fainted while breathing 11% oxygen without added CO_2, and two subjects fainted while breathing approximately 10% oxygen and sufficient CO_2 to prevent hypocapnia. The results of these four experiments are not included in the present series and will be discussed in a later section.

End-expiratory CO_2 tension. The successful compensation of CO_2 loss during anoxic hyperpnoea is illustrated in Fig. 4. When CO_2 was added in appropriate concentration to the inspired oxygen-deficient gas mixture, end-expiratory pCO_2 remained unchanged in five subjects and dropped by 1 mm Hg in the sixth (Table 1). By contrast, when no CO_2 was administered inhalation of 11% oxygen caused a fall in end-expiratory pCO_2 in

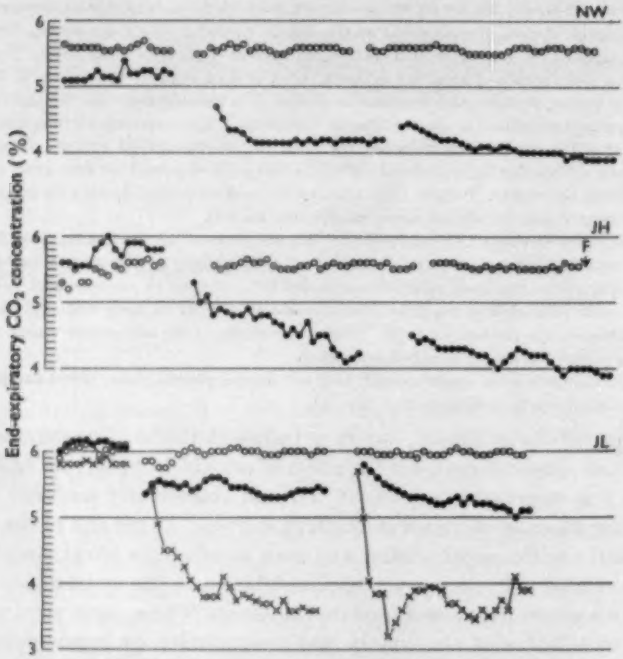

Fig. 4. End-expiratory CO_2 concentration recorded every minute during inhalation of 11% O_2 in N_2 (●) or of 10% O_2 in N_2 with sufficient CO_2 added to maintain the pre-anoxic level (○). The first part on the left of each record shows the end-expiratory CO_2 concentration during the last few minutes before the start of anoxia while the subject was still breathing room air. Gaps in the records are due to interruptions for the collection of urine specimens. An unsuccessful attempt to match the hyperpnoea of anoxia by slight deliberate overventilation with room air is also shown (×).

all subjects, the lowest level being attained at the end of the hour's exposure. The subjects were unaware that their pulmonary ventilation was increased during anoxia, whether or not CO_2 was added.

The pH of blood from the antecubital vein rose by 0·01–0·09 pH units when oxygen-poor gas without added CO_2 was breathed, but it did not alter during anoxia if CO_2 was added to the inspired gas mixture to avoid

the development of hypocapnia. The data suggest that in this manner alkalaemia was successfully prevented without causing acidaemia. In the absence of arterial blood samples, however, some uncertainty remained whether compensation had in fact been as accurate as was apparent.

TABLE 1. Changes in end-expiratory carbon dioxide tensions (mm Hg)

	Anoxia						Minimal voluntary overbreathing (air)		
	No CO_2 added			CO_2 added					
Subject	Initial	Final	Difference	Initial	Final	Difference	Initial	Final	Difference
PF	40	34	− 6	39	39	0			
CT	38	31	− 7	42	42	0	38	21	−17
JL	44	37	− 7	43	43	0	41	25	−16
JE	41	28	−13	40	39	−1			
NW	37	28	− 9	40	40	0			
JM	41	37	− 4	41	41	0			

Chemical changes in blood and serum. The great slowing of the blood flow in the arm during anoxia may itself have affected the composition of the blood, so that venous samples taken before, during and after anoxia were probably not strictly comparable. In general, there was little change: alterations in serum creatinine and urea were negligible, and no consistent changes in serum sodium were detected. Serum potassium increased slightly in anoxia with hypocapnia (mean 0·14 m-equiv/l., range 0–0·31 as it did during moderate hypocapnia without oxygen lack; but no consistent change was seen in anoxia without hypocapnia. Serum chloride fell by 2 m-equiv/l. (range 0–8) in both types of anoxia. Serum inorganic phosphate decreased during anoxia with hypocapnia (mean 0·4 m-equiv/l., range 0·2–0·6) but did not alter if CO_2 loss was compensated. Haematocrit and serum protein remained unchanged, in contrast to the findings of Asmussen & Nielsen (1945).

Volume and composition of the urine. Breathing an oxygen-poor gas mixture after water ingestion caused an increase of diuresis. The enhancement occurred in every subject and was, on average, of equal magnitude whether or not anoxia was associated with hypocapnia (Fig. 5).

Figure 6 shows the changes in sodium, potassium, bicarbonate, chloride, urea and creatinine excretion, and in urinary pH which took place at the same time. It also shows the calculated total excretion of osmotically active solute, [2(Na + K) + urea]. At the peak of diuresis the latter exceeded the corresponding control value by 32 μosmole/min when CO_2 loss during anoxia was prevented, and by 251 μosmole/min when hypocapnia was allowed to develop.

The data summarized in Figs. 5 and 6 were submitted to analysis of variance in order to separate differences between individuals and differences between procedures. The former were of no particular interest in

the present context. The significance of differences between procedures was assessed by Student's t test (Table 2).

Anoxia with hypocapnia and controls (I in Table 2) differed significantly during the first or second half hour of anoxia with respect to water ($P < 0.01$), sodium ($P < 0.05$; $P < 0.02$), potassium ($P < 0.05$), chloride

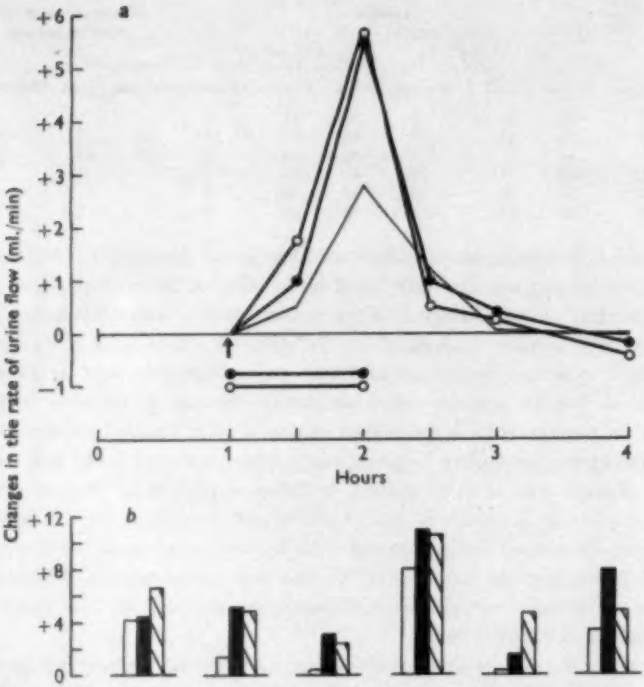

Fig. 5. *a*. Changes in rate of urine flow, mean values for six subjects. 500 ml. water drunk at time marked by arrow. Thin line, control; ● anoxia with hypocapnia; ○ anoxia without hypocapnia; duration of low-oxygen breathing shown by horizontal bars. *b*. Change in the rate of urine flow during period 3 (90–120 min); individual results: □ control; ■ anoxia with hypocapnia; ▨ anoxia without hypocapnia.

($P < 0.01$; $P < 0.05$), and bicarbonate ($P < 0.05$) excretion, and with respect to urine pH ($P < 0.01$).

Anoxia without hypocapnia and controls (II in Table 2) differed significantly during the first and second half hour of anoxia only with respect to water excretion ($P < 0.05$; $P < 0.01$). The observed increases in sodium and bicarbonate excretion and in urine pH were not significant ($P > 0.10$).

Anoxia with and anoxia without hypocapnia (III in Table 2) differed

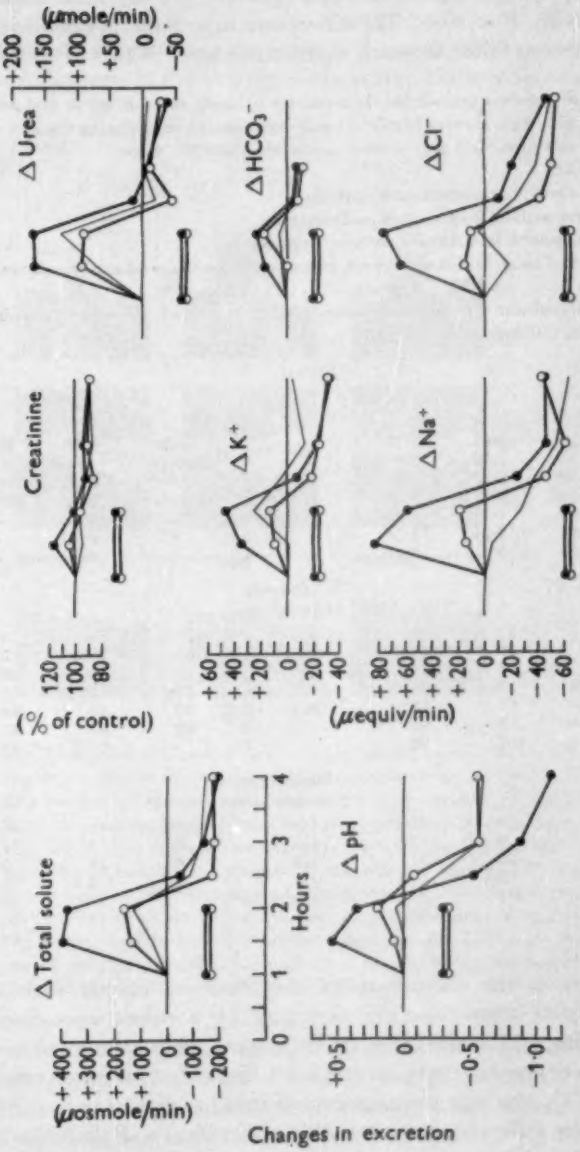

Fig. 6. Changes in 'total solute' excretion, calculated as [2(Na + K) + urea], and in urine pH, creatinine and urea output, and rates of excretion of potassium, sodium, bicarbonate and chloride ions. Mean values for six subjects. Thin line, control; ● anoxia with hypocapnia; ○ anoxia without hypocapnia. Duration of anoxia shown by horizontal bars.

significantly only with respect to urine pH ($P < 0.05$) and chloride excretion ($P < 0.05$; $P < 0.05$). The differences in sodium, potassium and bicarbonate excretion failed to reach significance level ($0.10 > P > 0.05$).

TABLE 2. Differences between procedures with respect to mean rates of water and solute excretion and urine pH. Initial rates (period 1) and changes occurring during the first and second 30 min intervals (periods 2 and 3) after ingestion of 500 ml. water.

Differences of means:

I, between Anoxia with hypocapnia and controls;

II, between Anoxia without hypocapnia and controls;

III, between Anoxia with and Anoxia without hypocapnia;

S.E. Standard error of mean of 6 observations, calculated from the residual error shown by analysis of variance.

M.S.D. Minimal significant difference between means at stated level of probability, calculated as $2t \times$ S.E. (10 degrees of freedom)

Period ...	1	2	3	Period ...	1	2	3
Water (ml./min)				pH			
I	0·10	0·42	2·45	I	0·20	0·56	0·31
II	0·51	1·24	2·65	II	0·18	0·09	0·17
III	0·59	0·82	0·20	III	0·02	0·47	0·14
S.E.	0·153	0·395	0·505	S.E.	0·211	0·126	0·212
M.S.D. 10%	0·39	1·01	1·29	M.S.D. 10%	—	0·32	—
5%	0·48	1·24	1·59	5%	—	0·40	—
1%	0·68	—	2·20	1%	—	0·56	—
Sodium (μequiv/min)				Chloride (μequiv/min)			
I	26	88	86	I	26	86	70
II	21	20	46	II	45	30	5
III	47	68	40	III	71	55	66
S.E.	30·5	23·9	20·6	S.E.	27·4	15·6	19·3
M.S.D. 10%	—	61	53	M.S.D. 10%	70	40	49
5%	—	75	65	5%	86	49	61
1%	—	107	92	1%	—	70	86
Potassium (μequiv/min)				Bicarbonate (μequiv/min)			
I	6	35	21	I	2	13	17
II	20	9	13	II	6	3	12
III	26	26	34	III	8	16	5
S.E.	11·5	9·7	11·3	S.E.	6·3	5·9	5·0
M.S.D. 10%	—	25	29	M.S.D. 10%	—	15	13
5%	—	31	35	5%	—	18	15
1%	—	—	—	1%	—	—	22

A complication in the comparison of the effects of anoxia with and without hypocapnia arose from the fact that by accident unconnected with the experimental procedures urine volume and sodium chloride excretion before exposure to anoxia (period 1 in Table 2) were greater on the days when CO_2 loss was compensated than when it was not, and for the urine flow this difference was statistically significant ($P < 0.05$). The results in Figs. 5 and 6 are expressed as differences from the initial rates, and it is impossible to decide with certainty whether the effects of anoxia

were enhanced or diminished by being superimposed on higher starting rates of excretion.

Absolute values for ammonium and hydrogen ion excretion showed no clear differences in the two types of anoxia. Differences were better defined

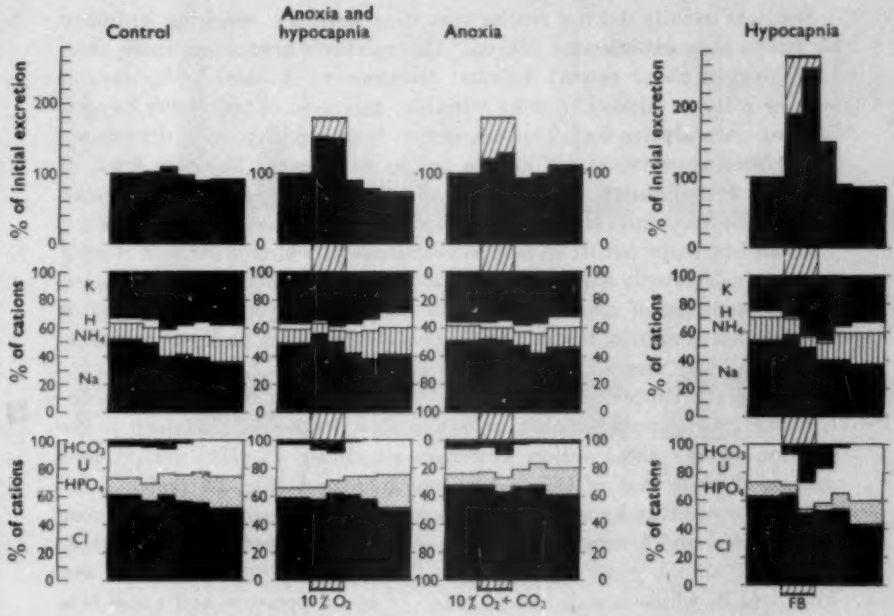

Fig. 7. Relative changes in cation and anion excretion. The top line of histograms shows changes in the excretion of all estimated cations ($Na^+ + K^+ + H^+ + NH_4^+$) as a percentage of their initial rate of excretion (period 1). The diagrams below show the fraction of the sum of cations represented by potassium, hydrogen ion, ammonium and sodium, respectively; and the fraction of the sum of cations balanced by bicarbonate, phosphate, and chloride respectively; U stands for 'undetermined' anions (sulphate and others) presumed to account for the difference. The results shown for control, anoxia with, and anoxia without hypocapnia are the mean values for six subjects; those for hypocapnia are mean values for two of the six who attempted unsuccessfully to match the hyperpnoea of anoxia by deliberately overventilating with room air (cf. Table 1).

when relative changes in the urinary cations were considered. In anoxia with hypocapnia the share of sodium and potassium in the total cation load increased at the expense of hydrogen ion and ammonium (Fig. 7), as it does, more markedly, during plain respiratory alkalosis. In anoxia without hypocapnia this was not the case.

Distress during anoxia

In the preceding sections only those experiments were included in which anoxia was subjectively well tolerated; if it was not, different effects on renal excretion were observed.

Subjects usually did not realize that they were not respiring ordinary air unless they experienced distress. Gas mixtures containing more than 11% oxygen never caused distress; reactions to 11 and 10% oxygen with or without added CO_2 were variable; mixtures of still lower oxygen content were always found unpleasant, at least initially, until drowsiness or euphoria supervened. If anoxia led to an episode, however brief, of malaise, apprehension, excitement, vertigo, or nausea, polyuria either failed to develop from the start, or was abruptly converted to oliguria.

In all but three out of sixteen experiments in which urine flow during anoxia was suddenly suppressed, this happened with little or no alteration in the excretion of creatinine. In all instances oliguria persisted after termination of anoxia, frequently for periods exceeding an hour; in water-loaded subjects diuresis would then reappear (Fig. 8).

Despite the suppression of diuresis the response of the kidney to concomitant hypocapnia remained intact in these instances, inasmuch as the fraction of the total cation excretion consisting of $(H^+ + NH_4^+)$ ions decreased while that of $(Na^+ + K^+)$ ions increased (Fig. 8*a* and *c*). According to whether hypocapnia was present or not, sodium excretion rose more or less, and in most experiments remained elevated during the whole period of anoxia even though urine flow became inhibited. In a few instances in which anoxia led to a fall of blood pressure and pulse rate sodium excretion diminished with the urine flow and remained depressed for a variable length of time (Fig. 8*d*). A much more definite disturbance of sodium excretion was seen after syncope.

Four subjects suddenly and without premonitory symptoms lost consciousness while breathing 10 or 11% oxygen. One felt too ill afterwards for observations to be continued, another could produce no urine during the next 4½ hr. In the remaining two the course of events was very similar and is illustrated in Fig. 9*a*. After syncope urine flow dropped to a very low rate. Simultaneously with the inhibition of diuresis the urinary concentration of sodium fell, and consequently renal sodium output dropped precipitously, not only in absolute amount, but also relative to other cations, with potassium becoming dominant (Fig. 9*a*). Recovery was still incomplete 2½ hr after the faint and return to air breathing.

It was found that if a subject fainted while breathing room air, e.g. during venepuncture, this also was followed invariably by prolonged antidiuresis and depression of sodium excretion. The effect of syncope was the

same whether it occurred before (Fig. 9*b*, *c*), during (Fig. 9*a*), or after (Fig. 9*d*) anoxia.

In two subjects (four experiments) who had fainted before starting to inhale oxygen-deficient gas mixtures containing no added CO_2 the normal

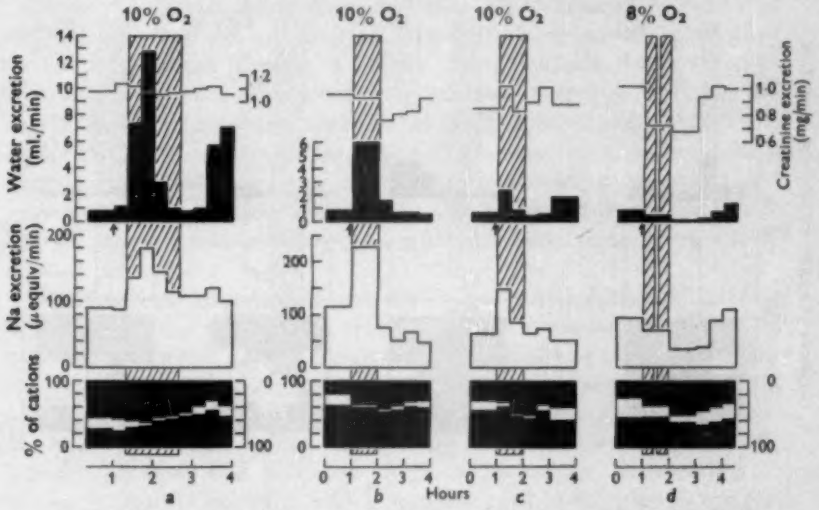

Fig. 8. Effect of distress during anoxia on urine excretion after water load. In each diagram, from above downward: thin line, creatinine excretion; ■ rate of urine flow; □ rate of sodium excretion: note different ordinate scales for *a*, and for *b*, *c*, *d*. Rectangles below, ion distribution, from above downwards: K^+ (black), $NH_4^+ + H^+$ (white) and Na^+ (black), as fractions of total cation excretion. Duration of anoxia shown by hatched fields. No CO_2 was added to the oxygen-poor gas mixtures.

Subject in experiment *a* drank 1000 ml. water at time marked by arrow; after 45 min of anoxia he became agitated and started to sweat, but calmed down again almost at once and continued on low O_2. Experiments *b*, *c* and *d* show effect of anoxia of increasing severity on excretory pattern of another subject who drank 500 ml. water on each occasion immediately before period of anoxia. In experiment *b* she remained quietly seated throughout exposure; in experiment *c* polyuria started to develop but was suppressed when subject moved to collect urine specimen during anoxia. Experiment *d* had to be interrupted and prematurely terminated, as shown, because of an abrupt fall in blood pressure and pulse rate. Subject experienced nausea and vertigo after return to air breathing but did not lose consciousness.

response to hypocapnia during anoxia was almost or completely absent (Fig. 9*c*, *d*). This appeared to be the consequence of the powerful inhibition of sodium excretion following syncope.

Urine flow and sodium output were not correlated with creatinine excretion on these occasions; nor did changes of sodium and water excretion go parallel to one another in all periods.

Abnormal constituents in the urine

Neither glucose nor protein were present in any of the urine specimens collected during this investigation.

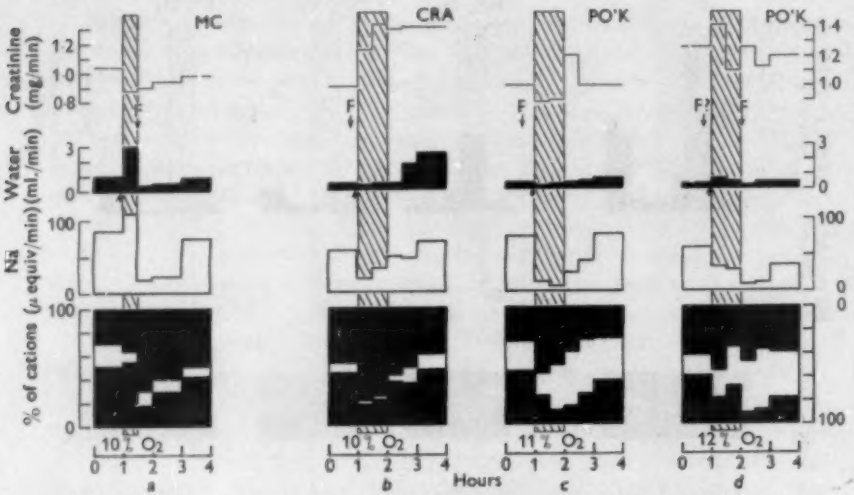

Fig. 9. Effect of syncope on water and sodium excretion. Arrangement as in Fig. 8. Duration of anoxia shown by hatched fields. In experiments *b*, *c* and *d* no CO_2 was added to oxygen-poor gas mixtures. Subjects MC and PO'K drank 500 ml. at times shown by arrows; subject CRA 150 ml.

In experiment *a* the subject fainted during anoxia. In experiments *b* and *c* the subjects fainted during venepuncture while breathing air normally approximately 10–15 min *before* start of exposure to anoxia. In experiment *d* the subject felt faint and nauseated but did not lose consciousness during venepuncture before start of anoxia. He fainted when blood was drawn 3 min after return to air breathing.

DISCUSSION

When air of low oxygen content is breathed the severity of the resulting anoxaemia is lessened if CO_2 is added to the inspired gas (Cormack, Cunningham & Gee, 1957). In order to induce the same degree of systemic oxygen lack in all experiments a mixture of lower oxygen content was therefore administered when CO_2 loss was being compensated. Since arterial blood was not analysed, no direct proof was obtained that the level of arterial saturation was in fact exactly the same in both types of experiment; but the outward signs of anoxia indicated that it must have been closely comparable. Cyanosis, pallor, drowsiness and impairment of bladder control were equally marked in the presence or absence of hypocapnia, nor did administration of CO_2 alleviate anoxia sufficiently to

prevent fainting. It was unlikely therefore that the different effects on renal function of anoxia with and anoxia without concomitant hypocapnia should have derived from primary differences in the intensity of oxygen lack.

Numerous authors have suggested that the increased excretion of sodium and potassium at simulated high altitude might be the result of respiratory alkalosis (Langley & Clarke, 1942; Ochwadt, 1947; Fishman *et al.* 1951; Axelrod & Pitts, 1952). By contrast, Berger *et al.* (1949) did not consider this possibility, and Selkurt (1953, 1954) thought that hypocapnia played some part in the observed changes, but that in addition oxygen lack directly impaired the tubular reabsorption of sodium and potassium. Most of these workers and also Asmussen & Nielsen (1945) stressed that a great increase in chloride excretion takes place during anoxia.

In the present experiments the rise of potassium excretion and the diminution in the excretion of $(H^+ + NH_4^+)$ ions relative to $(Na^+ + K^+)$ ions—changes which are characteristic consequences of respiratory alkalosis—disappeared when the development of hypocapnia during anoxia was prevented. The rise of chloride excretion also was no longer found, which suggested that it was in some way connected with hypocapnia. When hypocapnia was produced by forced breathing considerable increases in chloride excretion were indeed observed in the present investigation, and by others (Davies, Haldane & Kennaway, 1920; McCance & Widdowson, 1936). The phenomenon has no known explanation at present.

In general, the results presented in Fig. 6 and Table 2 appeared to show that moderately severe systemic oxygen lack, as such, had little, if any, direct effect on renal electrolyte excretion, particularly in view of the greater urine volume. However, this cannot be asserted with full confidence, because of the residual changes in sodium excretion, and also because the statistical analysis did not reveal an unequivocal distinction in this respect between anoxia with and anoxia without hypocapnia.

Acute anoxic anoxia outside the laboratory is always associated with hypocapnia. It was therefore of interest that in the present experiments anoxia did not interfere with the homoeostatic adjustments of electrolyte excretion which tend to restore acid/base equilibrium in respiratory alkalosis. This was true (except after syncope), even if anoxia led to oliguria and there was no over-all rise in electrolyte excretion.

As is shown in Fig. 5, anoxia with and without hypocapnia produced equal effects on urine flow, enhancing the diuresis after water ingestion. In the experiments with hypocapnia there was a greater increase in the excretion of solutes (Fig. 6), which might have been expected to cause a greater urine flow by their osmotic effect (Rapoport, Brodsky, West &

Mackler, 1949; Kleeman, Epstein & White, 1956). Interpretation is complicated by the accident that resting urine flow before anoxia was different in the two sets of experiments. The possible reasons for the increase in urinary volume in these experiments will be much more extensively discussed in the following paper (Currie & Ullmann, 1961) in connexion with the observation that urine flow may rise in any circumstance involving alteration of respiration, even in the absence of anoxia or hypocapnia. The experiments described in the present paper indicated that something other than hypocapnia may act to augment diuresis.

Silvette (1944*a*, *b*) has suggested that secretion of anti-diuretic hormone (ADH) might be impaired in anoxia. In the present investigation the diuretic effect of anoxia was found to be minimal in dehydrated subjects. If there was no concomitant hypocapnia it could be suppressed altogether by the intravenous injection of vasopressin (Ullmann, unpublished). The comparatively rare instances in which urine flow decreased during anoxia all followed episodes of distress, and the pattern of oliguria was typical of pituitary anti-diuresis. Evidently the neurohypophysis remained capable of discharging its hormone when suitably stimulated, despite severe anoxaemia, and the cells of the kidney of responding to it. It remains possible, however, that anoxia diminished the basal rate of hormone release from the gland.

On the basis of animal experiments Stickney, Northup & Van Liere (1946) came to the conclusion that mild anoxia led to polyuria and severe anoxia to oliguria. This appears to be true for human subjects, because severe anoxia tends to cause distress. In the present experiments the effect of distress on the excretory pattern during anoxia had at least two components. The first was inhibition of urine flow, to all appearances the consequence of a discharge of ADH from the neurohypophysis (Brun, Knudsen & Raaschou, 1945; Noble & Taylor, 1953). Oliguria during anoxia was always preceded by some 'emotional' disturbance, or by vertigo, or by the vasovagal syndrome. It was only rarely associated with a fall of creatinine excretion, and if it was, the latter returned to control levels much earlier than the urine flow. Oliguria always continued after the cessation of anoxia. It was, therefore, not analogous to the rapidly reversible anuria which is the result of paroxysmal renal vasoconstriction in intensely anoxic anaesthetized animals (Franklin, McGee & Ullmann, 1951; de Corral & Sálces, 1955). There was nothing to distinguish oliguria during anoxia from antidiuresis mediated by the posterior pituitary in non-anoxic states. It is highly probable that the oliguria accompanying mountain sickness (Stämpfli & Eberle, 1944) and dehydration at high altitude (Hunt, 1953) is likewise the result of neurohypophysial stimulation.

The second effect of distress was a profound depression of sodium excre-

tion which also would persist for an hour or longer after anoxia had ceased. This, unlike inhibition of urine flow, was never seen in distressed subjects unless they had fainted, or nearly fainted. In the only previously recorded instance of depression of sodium excretion following acute anoxia the subject had become apnoeic through forced breathing and finally lost consciousness (McCance, 1935). In the course of the present investigation it became clear that the stimulus was not antecedent anoxia as such, but vasovagal collapse, irrespective of whether this was brought on by systemic oxygen lack, or by 'emotion'.

Suppression of sodium excretion did not appear to be simply a consequence of the fall in urine flow after syncope. The two changes did not always follow the same time course; sodium excretion was depressed relative to other cations; and not only the total output but the urinary concentration of sodium fell. It was also probably not directly related to changes in renal haemodynamics, because these are known to recover rapidly after vasovagal fainting (Brun *et al.* 1945; de Wardener & McSwiney, 1951). A long-lasting depression of sodium output with similar characteristics has been observed in various other conditions involving a transient episode of hypotension, or of blood-pooling in the limbs (Assali, Kaplan, Fomon, Douglass & Tada, 1951; Chalmers, Lewis & Pawan, 1952), but by what mechanism the renal tubules are affected is uncertain. Even the combination of syncope and low arterial oxygen tension failed to cause anoxic damage in the kidney if absence of proteinuria and glycosuria after these episodes may be regarded as a sign of continued structural integrity.

SUMMARY

1. A study was made of the effects of acute anoxic anoxia with and without concomitant hypocapnia on the volume and composition of the urine of healthy human adults.

2. The respired gas mixtures contained 14–8 % oxygen, usually approximately 10 %. Exposure to anoxia lasted one hour.

3. The excretion of sodium, potassium, bicarbonate and chloride increased, and the ratio of $(H^+ + NH_4^+)$ ions to $(Na^+ + K^+)$ ions decreased if anoxia was accompanied by hypocapnia.

4. If during anoxia the development of hypocapnia was carefully prevented only trivial alterations in urinary solute output were found.

5. In most experiments anoxia led to polyuria and enhanced the diuresis following ingestion of water. This could only in part be attributed to a rise in solute excretion, since it occurred equally in the presence and absence of hypocapnia, i.e. of large and small changes in solute output. The findings were consistent with the hypothesis that in acute anoxia the basal rate of secretion of antidiuretic hormone (ADH) may be diminished.

6. If anoxia caused distress the subjects rapidly became oliguric. This appeared to be the result of a sudden discharge of ADH from the neurohypophysis.

7. Fainting during anoxia was followed by profound oliguria and long-lasting, specific depression of sodium excretion.

8. The changes in renal excretion which were associated with hyperpnoea, hypocapnia, distress and the vasovagal syndrome in anoxia were the same as those which accompany these extra-renal disturbances if they arise from causes other than systemic oxygen lack. It appeared therefore that the intrinsic responses of the nephrons to those agents which modify their excretory and regulatory function were not defective in anoxia.

I wish to record my gratitude to the many students and colleagues who acted as subjects. I want to thank Dr D. J. C. Cunningham for advice on the problems of continuous recording and control of alveolar CO_2 concentrations; and Drs Alison M. Fergusson, C. Todd, and J. F. Hale and Miss Ethel Cane for valuable assistance. The colorimeter, flame photometer and CO_2 analyser used were bought with a grant from the Central Research Fund of the University of London, for which I am much indebted.

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POLYURIA DURING EXPERIMENTAL MODIFICATIONS OF BREATHING

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In the course of experiments on 'the regulation of the blood's alkalinity' Davies, Haldane & Kennaway (1920) discovered that inhalation of CO_2-enriched air led to a marked rise in urine flow, and that voluntary overventilation resulting in loss of CO_2 from the body also caused diuresis. Both observations have been repeatedly confirmed but not yet satisfactorily explained. Barbour, Bull, Evans, Hughes Jones & Logothetopoulos (1953) showed that during hypercapnia only water excretion was increased, while the rate of urinary solute output was not greatly altered. In acute hypocapnia, by contrast, both water and solute excretion are augmented (McCance & Widdowson, 1936), and it has been suggested that the increase in urine flow is merely the consequence of raised urinary electrolyte excretion (Stanbury & Thomson, 1952; Robinson, 1954). However, increased solute excretion cannot be the only factor responsible for the diuresis, because very high rates of urine flow may also be encountered during or shortly after forced breathing at times when urinary solute excretion is not raised (Ullmann, unpublished).

Changes in the rate of urine flow, which in pattern and time course closely resemble those occurring during CO_2 inhalation or during forced overbreathing, were found also in acute anoxic anoxia. There was usually a simultaneous rise in the excretion of electrolytes. If anoxia was produced by inspiring a gas mixture low in oxygen but containing sufficient CO_2 to prevent hypocapnia from developing there was little change in electrolyte excretion, but the urine flow still increased (Ullmann, 1961).

Almost the only factor common to CO_2 inhalation, forced overbreathing, and acute anoxic anoxia is increased ventilation of the lungs. It was therefore of interest to determine whether hyperpnoea as such had some influence on the rate of urine formation in the absence of any concomitant changes in blood gas tensions. We first approached this problem by observing the renal effects of voluntary overventilation with air to which sufficient CO_2 was added to prevent respiratory alkalosis.

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METHODS

Subjects. The subjects were healthy adults of both sexes, aged 18–45 years. Excepting the authors, they had not taken part in previous experiments and did not know the purpose of the present ones. They were asked not to drink tea or coffee during the 12 hr period preceding and not to smoke during experiments. Apart from this, their activity, diet, and fluid intake was not controlled unless otherwise stated. The same individuals took part in 'control' and serial 'experimental' runs, which were performed in randomized sequence, and always at the same time of day. Details of experimental routine varied, and will be given in the text.

Recording of respiration. The apparatus used for administering various gas mixtures and for continuously sampling and analysing end-expiratory air was described in the preceding paper (Ullmann, 1961). Some additions were made for the present investigation.

The wide-bore tubing conveying expired air from the valve was connected via a moisture trap to a gas flowmeter having a resistance of less than 50 mm H_2O at a steady flow rate of 200 l./min (Model C4, made by Messrs Parkinson and Cowes). The exit tube from the CO_2 analyser was joined to the inlet tube of the flowmeter so that the side-tracked end-tidal air sample was returned to the main stream of expired air going to the meter. A tambour carrying a pointer was connected to the Rahn–Otis sampler and served to record respiration rate on a smoked drum. In this manner continuous records of respiration rate, total pulmonary ventilation and tidal volume were obtained, together with the minute-to-minute values of end-expiratory CO_2 concentration.

Recording of intraoesophageal pressure changes. Intraoesophageal pressure changes during the respiratory cycle were recorded either by means of a water-filled catheter, as described by Dorhnorst & Leathart (1952), or in later experiments with an air-filled balloon, 15 cm long and having a diameter of 0·6 cm (Mead, McIlroy, Selverstone & Kriete, 1955). Respiratory pressure fluctuations were measured with a capacitance manometer (Southern Instruments Co.) and recorded by pen writer on very slowly moving paper or by oscilloscope and camera on fast-moving paper.

Experimental modifications of breathing

Hyperpnoea. The volume of ventilation was increased by voluntary forced breathing paced by a metronome, by inhalation of 5–6 % CO_2, or by inhalation of 10–11 % oxygen.

Breathing against resistance. The minimal resistance of the external respiratory circuit consisted, on the inspiratory side, of the combined resistances of the thin rubber flap on the inspiratory side of the Siebe–Gorman valve and the wide-bore tubing leading to it from the gas reservoir; and on the expiratory side of the combined resistances of the expiratory rubber flap on the valve, of the flowmeter, and of the connecting tubing. The resistances of tubing and flowmeter were negligible and almost the entire resistance due to the basic external respiratory circuit resided in the rubber flaps of the valve. Added external resistances were always placed in the *inspiratory* pathway. External expiratory resistance was kept constant at the minimal level.

Added inspiratory resistances were of two kinds, non-elastic and elastic; the former were graded low (L), moderate (M), and high (H). The low non-elastic resistance was simply the minimal inspiratory resistance of the circuit. Moderate resistance was produced by placing three rubber flaps instead of one on the inspiratory side of the Siebe–Gorman valve. The high resistance was an 8 cm length of glass tubing inserted into the external airway approximately 30 cm above the inspiratory valve. Depending on the size of the subject, tubes with an internal diameter of 3 or 4·5 mm were used.

When breathing against an added external elastic resistance (negative-pressure breathing) the subjects inspired not from the usual reservoir bag but from a ventilated tank of 198 l. capacity in which the pressure was kept 12–15 cm H_2O below atmospheric (T). The procedure

differed from 'negative pressure breathing' as ordinarily understood in that expiration was to the atmosphere through the usual expiratory pathway, and not back into the evacuated tank. The same Siebe–Gorman valve was used as in all other experiments.

Urine specimens. Timed samples were voided into graduated cylinders. These contained liquid paraffin if urinary pH or bicarbonate content was to be determined. Subjects unable to empty the bladder completely at specified times were excluded. Estimations of sodium, potassium and creatinine concentration, and of the pH and total osmolarity of the urine were carried out by the methods listed in the preceding paper (Ullmann, 1961). No blood samples were taken, in order to avoid accidental 'emotional' antidiuresis resulting from venepuncture.

RESULTS

Sixteen paired preliminary tests were carried out with the subjects seated; in eleven of these the volume of urine excreted when the subjects deliberately overventilated exceeded considerably the urine output in control runs. Though not conclusive in themselves these results suggested a possible association of hyperpnoea and polyuria which was partially masked by other factors. It was decided to conduct further experiments with the subjects recumbent, because Barbour *et al.* (1953) had emphasized that inhalation of CO_2 led to diuresis only when the subjects were supine but not when they were seated.

Experiments on recumbent subjects

Six subjects were studied of whom only two had taken part in the preliminary tests. They came to the laboratory 2 hr after breakfast and lay down on a couch with the head raised just sufficiently to allow them to read. They were given nothing to drink during the entire period of observation. Urine samples were collected at 15 min intervals, the subjects getting up to void.

The number of procedures used to induce hyperpnoea, and the number of experiments with one individual varied. The start of any experimental procedure was delayed, usually for 2–3 hr, until the spontaneous initial diuresis which occurs on changing from the upright to the recumbent posture had subsided (Barbour *et al.* 1953). Only after three or more consecutive periods of falling rates of urine flow was forced breathing (or some other test procedure) begun; it was then continued for three urine-collecting periods (45 min), and followed by further observation for at least 30 min afterwards.

In experiments involving forced overbreathing or anoxia the CO_2 content of the inspired gas was continuously adjusted in the manner described in the preceding paper (Ullmann, 1961). The aim was to maintain the end-expiratory pCO_2 constant at the value found before the change of respiration, or preferably 2 mm Hg above this level. This was readily achieved in anoxia. During the first 10–15 min of voluntary overventilation, however, fluctuations in the end-expiratory CO_2 level could not always be prevented, because of the much more rapid and unpredictable rate of CO_2 loss from the blood. But it was always possible to maintain the *mean* end-expiratory pCO_2 during hyperpnoea within $\pm 0{\cdot}5$ mm Hg of the mean resting value. End-expiratory pCO_2, respiratory minute volume, and respiration rate were recorded from the time the initial spontaneous diuresis began to decline until the end of observations.

Figure 1 shows the changes in the rate of urine flow elicited by voluntary overbreathing in twelve experiments on six subjects. Mean respiratory

minute volumes during forced breathing were 1·9–4·5 times greater than the corresponding mean resting values.

In nine of the experiments illustrated the subjects were instructed to overventilate slowly and deeply, and in all these hyperpnoea led to a rise in urine flow which was of the same order of magnitude as, or greater than, the initial 'spontaneous' recumbency diuresis. In three further experiments the subjects achieved similar increases of ventilatory minute volume

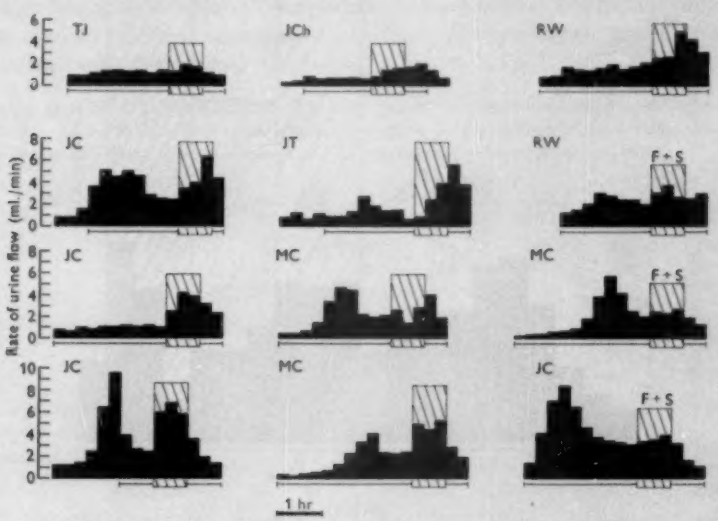

Fig. 1. Effect of voluntary forced breathing without hypocapnia on the urine flow of recumbent subjects. Subjects started breathing through the valve at zero time. No water given before or during period of observation. Period of overbreathing, shown by hatched fields, began after initial recumbency diuresis had subsided. pCO_2 kept constant. F + S, fast and shallow breathing.

by breathing rapidly with a low tidal volume; on these occasions, however, no definite polyuria developed and only a reversal of the downward trend of excretion was found.

Figure 2 shows a comparison between forced breathing and CO_2 inhalation in the course of a single experiment. In Fig. 3 the effects of forced breathing, CO_2 inhalation, and inhalation of 10% oxygen are compared, one subject being tested on three separate occasions. Despite high urine volumes at the start, the diuretic effect of all three experimental procedures was marked, and of similar magnitude. In the examples shown urine flow increased from 1·80, 2·35, and 2·20 ml./min during the last preceding period to 6·75, 6·35, and 9·55 ml./min during hyperpnoea. On all three occasions it fell off again rapidly as soon as normal breathing

recommenced. Total solute excretion rose at the same time, from 0·75, 0·80, and 0·77 m-osmole/min to 1·12, 1·34, and 1·95 m-osmole/min, about 3/4 of the change being accounted for by electrolytes [2(Na+K)]. There were no obvious changes in urine pH or creatinine excretion. These findings were typical of all similar experiments.

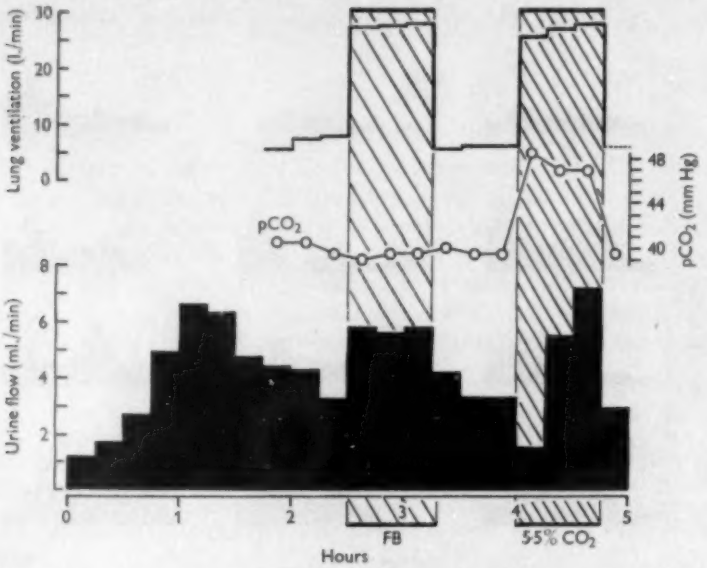

Fig. 2. A single continuous experiment showing in succession the spontaneous diuresis of recumbency, the polyuria elicited by forced breathing (FB) without hypocapnia, and polyuria elicited by CO_2 inhalation (5·5 % CO_2). pCO_2, end-expiratory CO_2 tension. The subject lay down and started valve breathing at zero time: duration of experimental procedures shown by hatched fields. He drank 150 ml. water 2½ hr before zero time, but had no other fluid since the previous evening and none during the experiment.

The polyuria which developed during hyperpnoea was not related in magnitude to the increase of respiratory minute volume or change in end-expiratory pCO_2. Nor could a definite correlation between the changes in rate or depth of breathing and the rise of the diuretic response be established, even though it appeared that hyperpnoea was relatively ineffective when breathing was shallow (Fig. 1). Deliberate fast and shallow respiration gives rise to a feeling of suffocation and is difficult to maintain for long periods. It was not clear whether this circumstance or the fact that the tidal volume was kept small was responsible for the diminished effect (Table 1).

The response of an individual to the same procedure was not constant from day to day. If the spontaneous diuresis on lying down was large the polyuria later induced by hyperpnoea was usually also large, and conversely (Fig. 1). The incidence and size of recumbency diuresis was itself variable, depending primarily on the subject's state of hydration, but also on other less obvious influences. In order to determine whether changes in the rate of urine flow on lying down would tend to become enhanced or suppressed with repetition of experiments, each of four additional subjects was studied on four consecutive occasions. No systematic trend became apparent with the limited number of trials.

TABLE 1. Change in urine flow during hyperpnoea. Subjects recumbent. Highest rate during or immediately after experimental procedure as percentage of rate of flow during last period preceding altered respiration. Corresponding values for control runs

Procedure	Subject					
	TJ	JCH	JT	RW	MC	JC
Control	82	115	112	67	100	165
	80	103	129	81	100	145
	—	—	—	70	—	97
Forced breathing: slow and deep	129	325	842	233	184	274
	—	—	—	—	134	230
	—	—	—	—	205	174
	—	—	—	—	—	420
Forced breathing: fast and shallow	—	—	—	185	122	128
Breathing 5–6 % CO_2	—	159	273	—	300	408
	—	—	—	—	—	216
	—	—	—	—	—	364
Breathing 10–11 % O_2	133	—	—	231	474	425
Normal breathing through valve	70	170	250	170	338	235

In all experiments hitherto discussed the subjects breathed air normally through the valve and mouth-piece from the moment they lay down. It was found that, if they started to breathe through the valve only after the spontaneous recumbency diuresis had subsided, valve-breathing by itself led to renewed polyuria. Two examples of 'valve diuresis' are illustrated in Fig. 4. One of them shows that if a subject continued to breathe through the valve the polyuria gradually subsided. The rise of flow was comparable in every way with spontaneous 'recumbency' diuresis and also with the response elicited by 'hyperpnoea'. However, the rate of ventilation was not increased by valve-breathing. This suggested that the operative factor during valve-breathing and in the various types of hyperpnoea might be a mechanical change in the thorax associated with the increased airway resistance. To investigate this possibility the intrathoracic pressure and its variation during the respiratory cycle was recorded in all later experiments.

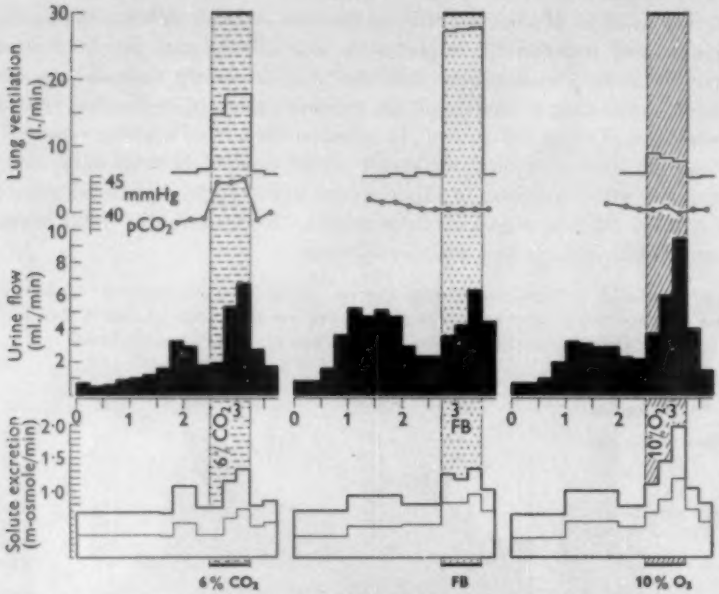

Fig. 3. Comparison of the effects of CO_2 inhalation (6 % CO_2), forced breathing (FB) and anoxia (10 % O_2) on urine flow. Three experiments on one subject, who on all occasions lay down at zero time and immediately started to breathe through the valve. Duration of experimental procedures shown by hatched fields. Abscissa, hours. pCO_2, end-expiratory CO_2 tension. Lower histograms show rate of total solute excretion. The thin line indicates electrolyte output, calculated as 2(Na + K). 200 ml. milk 2 hr before start of observations on all occasions, but no other fluid during 18 hr preceding and none during the experiments.

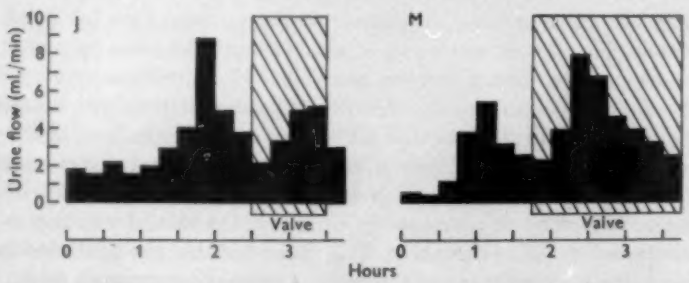

Fig. 4. Polyuria as the result of recumbency, and of breathing through the valve and mouthpiece used to administer gas mixtures. Except during periods shown by hatched fields the two subjects breathed normally without valve; they were supine from zero time onward. No fluid given.

When the subjects began breathing through the valve at the time they lay down 'valve diuresis' and spontaneous recumbency diuresis merged, and later subsided together. Urine flow would rise again subsequently only with further modification of respiration (Figs. 1, 2, 3).

Experiments on non-recumbent subjects

It was found that in non-recumbent subjects, also, water excretion was consistently enhanced during experimental modifications of respiration, provided they were given water to drink before the period of altered breathing. Two series of experiments were performed in which the subjects were seated. In the first the influence of altered breathing on the diuretic response to a small initial water load was tested; in the second its effect on a moderate, but long-maintained diuresis.

Effect of altered breathing on the excretion of a single small water load

Eight subjects were studied including three who had taken part in earlier tests. The subject emptied his bladder immediately after the introduction of a water-filled polythene catheter into the oesophagus. The first urine sample was collected 60 min later and the subject then drank 500 ml. of water. The next hour was subdivided into 2 urine collecting periods of 30 min and was occupied by the test procedure, if any. There followed a final period of 30 min, the catheter being withdrawn at the end. The subjects were seated throughout, but stood up to void.

'Control' observations were of two kinds: (*a*) the subject breathed air normally without valve during the whole 2½ hr (C); (*b*) the subject breathed air normally, but through the valve and mouth-piece, i.e. through a low non-elastic external airway resistance (L). Valve-breathing was begun 15 min after the start and continued till 15 min before the end of the 2½ hr of observation. The valve was used for the same length of time if breathing was experimentally modified during the second hour.

The following experimental procedures were used: (*a*) voluntary overventilation, pCO_2 kept constant as previously described (FB); (*b*) inhalation of 10 % oxygen in nitrogen, pCO_2 kept constant ($-O_2$); (*c*) inhalation of 5–6 % CO_2 in air (CO_2); (*d*) breathing against a high, non-elastic inspiratory resistance (H). Except in one single experiment, which was rejected, breathing against external resistances did not cause a change in the minute volume of ventilation, nor in the end-expiratory pCO_2. It was therefore not necessary to add CO_2 to the inspired air in these experiments.

Except for brief interruptions during voiding, and while the calibration was checked, the pressure changes in the oesophagus were recorded continuously for 2½ hr, including at least 15 min of normal respiration before and after the valve was in use. The amplitudes of all recorded breaths were measured (Dornhorst & Leathart, 1952).

Water excretion. The percentage of the water load excreted in 90 min in excess of the initial urine flow was calculated and results from all individuals in a group were averaged. All modifications of breathing augmented diuresis, there being little difference in the effectiveness of the four procedures. Merely breathing air through the valve produced an effect almost as great as that found when additional respiratory manœuvres were employed (Fig. 5).

The results were submitted to analysis of variance and the significance of the differences was assessed by Student's t test in the manner of the example illustrated in the previous paper (Ullmann, 1961). The differences

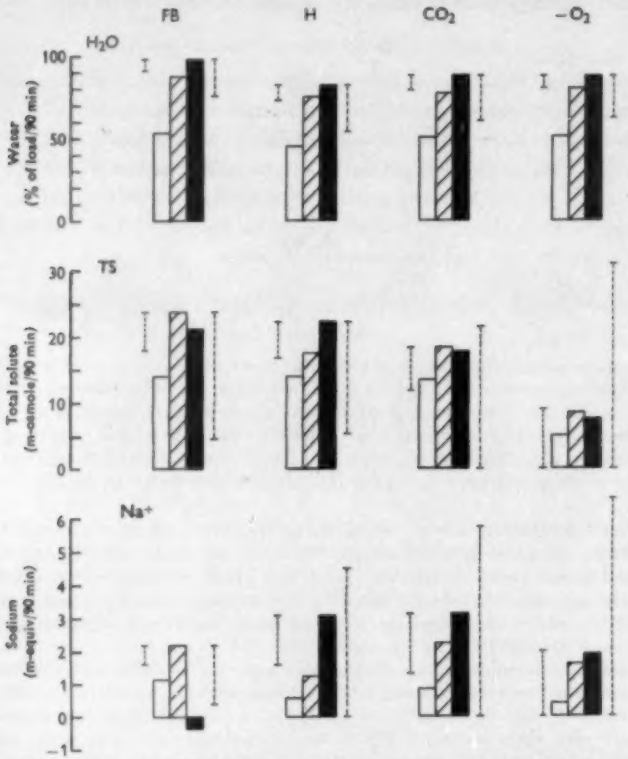

Fig. 5. Effect of altered breathing on the excretion of water, total urinary solute and sodium during 90 min after drinking 500 ml. water. Average values for groups of subjects calculated as excess over the control rates before water ingestion. The broken vertical line to the left of each group of means shows S.E. of mean; that on the right the minimal significant difference at the 5% probability level. □, control, normal breathing without valve. ▨, control, normal breathing through valve used in experiments. ■, experimental procedures: FB, forced breathing, pCO_2 kept constant (six subjects); H, high external inspiratory resistance (8 subjects); CO_2, breathing 5–6% CO_2 (6 subjects); $-O_2$, breathing 10% oxygen in nitrogen, pCO_2 kept constant (5 subjects).

in water excretion between control and experimental runs were significant statistically with each of the experimental procedures ($P < 0{\cdot}02$ or $P < 0{\cdot}01$); differences between experimental runs and control runs in which the valve was used were not; and in three out of the four groups the

difference between control runs with and without the valve was significant ($P < 0.05$ or $P < 0.01$).

Solute excretion. During all experimental procedures as well as during simple valve breathing the excretion of osmotically active solute in the 90 min after water ingestion was greater than during the control diuresis (Fig. 5). The excretion of sodium was also greater, except during forced breathing. Potassium excretion was slightly depressed by CO_2 inhalation but remained unaffected by all other procedures. In contrast to the augmentation of diuresis, however, the changes in solute excretion were not statistically significant, even at the peak of the diuresis when the differences were largest. There were no statistically significant differences in the initial excretion rates of water and solute which were used as the reference level.

In repeated experiments on one subject 37·5, 94·5, and 111·2% of the water load, on average, was excreted respectively in five control runs without valve, four control runs with valve and four experiments in which each modification of breathing was used once; the differences between the first and both other means being significant ($P < 0.01$).

Effect of altered breathing on maintained water diuresis

Five subjects were studied, two of whom had taken part in earlier experiments. They drank 7 ml. water/kg body weight at zero time; the balloon for recording intraoesophageal pressure was introduced at the same time. Subjects unable to swallow it without distress or retching could not be used.

The water load was maintained for 4½ hr by drinking every 30 min as much fluid as was excreted. Urine was collected at 30 min intervals throughout. If 1½ hr after zero time no diuresis had developed observations were not continued; if diuresis was satisfactory test procedures were begun 2 hr after zero time and continued for 2 hr; then followed a final 30 min without procedure.

During the first 2 hr the subjects always breathed normally without valve. No experiments involving forced overbreathing or low oxygen inhalation were carried out because of the long duration of the 'experimental' stage. Test procedures were chosen with the intention of producing graded increases in intrathoracic respiratory pressure fluctuations. They were, in addition to two control runs per subject in which breathing remained normal throughout: (*a*) breathing through a low, moderate or high external airway resistance (L, M, H), the 'low' resistance being the usual valve; (*b*) 'negative pressure breathing' (T), i.e. breathing from a tank in which the pressure was 12–15 cm H_2O below atmospheric; (*c*) breathing 5–6% CO_2. The order was randomized and the usual precautions observed.

In addition, observations were made with the subjects breathing normally without valve but supine (S) during the 2 hr 'experimental' period. This was done in order to compare quantitatively the excretory changes associated with alterations of respiratory pattern and the better known phenomenon of spontaneous recumbency diuresis. At all other times in this group of experiments the subjects were seated, and stood up to pass urine.

Water excretion. The magnitude of the diuresis in the first 2 hr varied widely even in the same subject, as in Fig. 6. Figure 6 shows that all experimental procedures enhanced the diuresis in this subject, except

negative-pressure breathing, which he found suffocating and which caused a transient antidiuresis. Usually all the procedures led to a rise in the urine volume, as is shown in the collected results in Fig. 7. In preparing Fig. 7 the highest rate of urine flow during the preliminary 2 hr was taken as the basis of comparison, and the mean rate (columns) and highest rate (circles) during the experiment expressed as a percentage of this. Points or columns

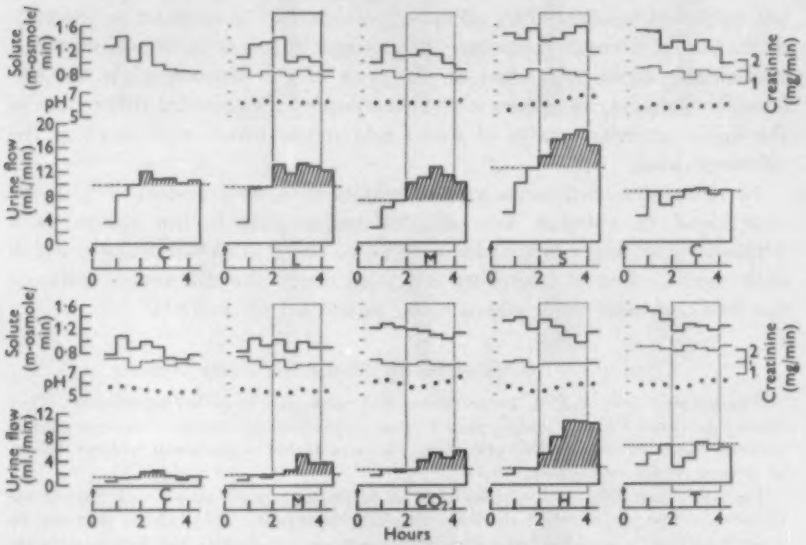

Fig. 6. Effect of altered breathing on maintained water diuresis. Ten experiments on subject R. In each record, from above downward: total solute excretion (heavy line); creatinine excretion (thin line); urine pH (●); rate of urine flow (histogram). The thin vertical lines show duration of experimental procedures. C, control; L, M, H = low, medium, high external airway resistance; S, supine, normal breathing; CO_2, breathing 6 % CO_2; T, breathing from tank evacuated to 150 mm H_2O below atmospheric pressure.

Broken horizontal lines correspond to highest rate of urine flow (= 100 %) during first 2 hr after drinking water load (arrow). Hatched areas show excretion in excess of this during second 2½ hr. Whatever the size of the initial diuresis, in the later stages urine flow increased more than in control runs during all experimental procedures except negative pressure breathing (T) which in this subject led to anti-diuresis.

above the 100 % line show increased diuresis during the second 2½ hr of observation. On the three occasions where external resistances (L, M, H) did not augment diuresis the initial diuresis was close to a maximal rate which could not be exceeded in this subject even if he was given much larger water loads. The enhancement of water excretion in the remaining

experiments was not large on a percentage basis, but owing to the high rates of urine flow prevailing at the start the additional volume of fluid eliminated in the course of 2½ hr was appreciable.

The significance of differences in water excretion in control and experimental runs was assessed by Student's t test, using the method of paired experiments. The enhancement of diuresis was significant in the statistical sense only with two of the procedures. These were, breathing through a 'moderate' external airway resistance (+40 %, S.D. ± 28·3; $P < 0{\cdot}05$), and lying supine (+27 %, S.D. ± 5·5; $P < 0{\cdot}02$). Too few experiments were carried out in this series for a valid comparison of the effectiveness of

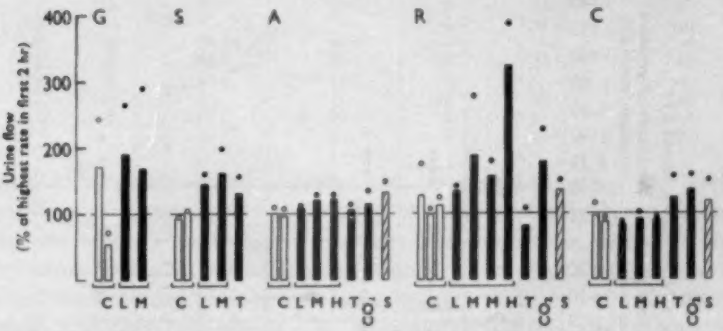

Fig. 7. Effect of altered breathing on maintained water diuresis. Summary of results. Mean rate of urine flow (columns) and highest rate of urine flow (circles) as percentage of highest rate (100 %) during the first 2 hr of observation. □, controls (C); ■, altered breathing (L, M, H, T, CO_2); ▨, supine, normal breathing (S).

different respiratory manœuvres, but it was clear that no one procedure led to a strikingly larger polyuria than the rest.

Solute excretion. During all experimental procedures total urine solute output remained constant or fell. Urine pH remained constant or underwent only such fluctuations as were found during control runs. Similarly, urinary creatinine output did not differ systematically from controls and did not parallel changes in rate of urine flow.

Respiratory oesophageal pressure variation and its relation to the changes in excretion

The amplitude of the respiratory pressure variation in seated subjects at rest averaged 39·0 and 40·5 mm H_2O during normal breathing in the two series of determinations. The amplitude was increased by all experimental procedures. Breathing through the valve caused an average increase of 10 mm H_2O in the first series and of 14·8 mm H_2O in the second. The larger change in the longer experiments was probably due to increasing

valve resistance owing to progressive deposition of moisture on the rubber flaps.

Other modifications of breathing were associated with greater average changes in amplitude. The scatter of individual results in one series of experiments is recorded in Fig. 8; it was similar in the other. Figure 8 also shows that the proportion of the respiratory cycle during which the oesophageal pressure was lower than in expiration became longer with all experimental procedures employed except negative pressure breathing (T). This had the opposite effect, prolonging the expiratory pause.

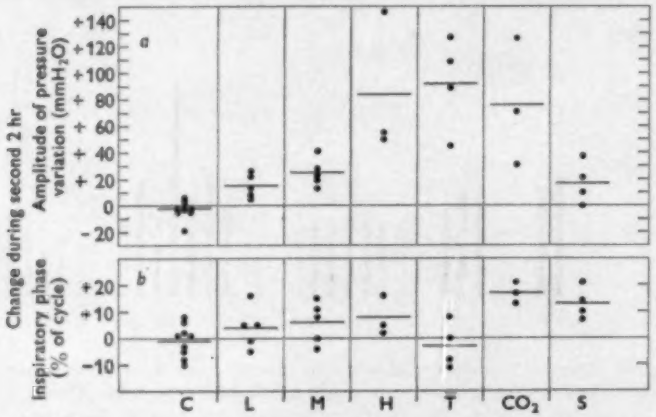

Fig. 8. Change in (*a*) amplitude of respiratory pressure swing and (*b*) duration of inspiratory phase of cycle during second 2 hr of observation compared with mean value during first 2 hr and final 30 min. Each dot gives the average value for one subject; bars the group averages. Same experiments as in Fig. 7.

In the supine posture, with the subjects breathing normally without valve (S), both amplitude of pressure swing and duration of inspiratory phase were increased, the former almost to the same extent as, and the latter rather more than when the subjects breathed through a moderate external airway resistance (M) while seated. As is shown in Table 2, the enhancement of diuresis by various respiratory manœuvres and the changes in intrathoracic (oesophageal) pressure fluctuations accompanying them were not directly related quantitatively. Water excretion was increased by valve-breathing when the pressure fluctuation was increased by 10–15 mm H_2O, i.e. by approximately 25% of the resting amplitude. When 10% oxygen was breathed or a moderate airway resistance was used there was an increase of 25–30 mm H_2O, or 50–60% of the resting amplitude of the pressure swing, and the diuretic effect was somewhat larger; but the still greater changes associated with CO_2 inhalation, negative-pressure breathing

(T) or high airway resistances (H) did not lead to further, proportionately greater excretory changes. Duration of inspiratory negativity, or various other functions involving both amplitude and duration of the

TABLE 2. Relation between respiratory and excretory changes. Average *increase* over control values

	Single water load			Maintained diuresis		
Procedure	No. of expts.	Δ Oesophageal pressure variation (mm H_2O)	Δ Excretion: (% of water load)	No. of expts.	Δ Oesophageal pressure variation (mm H_2O)	Δ Excretion: (% of reference value)
L	8	10·0	30·3	5	14·8	27
M	—	—	—	5	24·8	40
$-O_2$	5	26·0	36·5	—	—	—
CO_2	6	66·0	37·4	3	76·0	42
FB	6	92·0	44·7	—	—	—
T	—	—	—	4	92·0	10
H	8	141·0	36·1	3	81·0	77
S	—	—	—	3	20·8	27

pressure change correlated no better with the size of polyuria. In the first two series of experiments insufficiency of water in the body might have been the reason why diuresis did not continue to increase with increasing respiratory changes; but during maintained water diuresis a third of the water load remained to be excreted, on average, at the end of every 30 min period, and therefore the lack of quantitative correlation between the excretory effects and all measured respiratory variables must have had some cause other than progressive dehydration.

DISCUSSION

The experiments described gave no direct information about the immediate cause of the rise of urine flow which accompanied modifications of breathing. It seemed very unlikely that the polyuria was of 'emotional' origin because it occurred in a large number of subjects, all but two of whom knew nothing about the purpose or probable outcome of the experiments; on the rare occasions when a subject felt uncomfortable or apprehensive, urine excretion was suppressed and not enhanced. It was thought that the psychological effect of a raised airway resistance, even if not consciously appreciated, might result in overbreathing, but no evidence of this was found and the rise of urine flow could thus not be attributed to respiratory alkalosis.

The question whether the primary effect of altered breathing was on solute or on water excretion was important. Solute excretion increased when respiration was modified in some, but not in all experiments. If it did, the peak rates of solute excretion amounted to less than twice the resting rates, in contrast to manifold simultaneous changes in the rates of

urine flow; nor were increases in total solute or sodium excretion significant statistically. Nevertheless, the possibility that the increase of urine flow was an osmotic diuresis could not be ruled out definitely on these grounds alone, because it is unknown how much water is the minimum necessary for excretion of a given amount of solute by subjects in normal fluid balance.

It seemed possible that some of the change in solute output was the consequence rather than the cause of the change in urine flow. Solute excretion increased if the urine flow rose rapidly from a low initial level, that is, in circumstances when sodium and urea previously accumulated in the distal tubules and medulla in high concentration are flushed into the urine. For instance, the increase of urine flow which followed the drinking of 500 ml. water in control experiments was always accompanied by an increase in the excretion of solutes, although both rise of urine flow and rise of solute output were smaller than during experimental procedures. Furthermore, in the last series of tests, in which respiratory manœuvres were not started until some time after water diuresis was fully established, only the initial development of diuresis was accompanied by an increase of solute excretion; but when subsequently, during altered breathing urine flow rose still higher there was no further increase, and often a decrease, of solute output. This showed clearly that the enhancement of water excretion associated with altered respiration was not dependent on an augmented loss of solute.

All alterations of breathing which led to polyuria caused an increase in the amplitude of the respiratory variation of intrathoracic pressure. This might have given rise to circulatory changes which in turn affected glomerular filtration. This was not measured in the present experiments, and may or may not have been altered; but one would expect commensurate changes of solute and water excretion as the result of increased filtration, and these were not found.

Several features of the polyuria suggested that it might have been the result of some diminution of circulating anti-diuretic hormone (ADH). There was usually a delay of 20–30 min after the start of altered breathing before urine flow began to rise, although in recumbent subjects this was not invariably seen. As already discussed, changes of respiration promoted 'water' rather than 'solute' excretion. They had little or no influence on urine flow when subjects were dehydrated, or in circumstances likely to evoke an 'emotional' discharge of ADH. The higher the initial water load the smaller, in relative terms, was the enhancement of water excretion caused by altered breathing.

Recent careful work by Murdaugh, Sieker & Manfredi (1959) and the observations of Boylan & Antkowiak (1959) have made it virtually certain that the diuresis which develops in man and dog during negative-pressure

breathing (Sieker, Gauer & Henry, 1954) is due to lack of circulating ADH. Henry, Gauer & Reeves (1956) and Henry & Pearce (1956) have assembled much cogent evidence for their hypothesis that the secretory activity of the neurohypophysis may become reduced by inhibitory afferent nerve impulses which travel in the vagi and arise in deformation receptors situated in the left atrium. These are believed to be stimulated by distension, negative-pressure breathing leading to diuresis because a larger volume of blood fills the left atrium when intrathoracic pressure becomes more negative.

The findings reported in the present paper are consistent with the hypothesis that altered breathing affected urine excretion by the same or a closely similar mechanism. In all modifications of breathing studied by us intrathoracic pressure was lowered intermittently and not continuously, as in the experiments of Sieker *et al.* (1954). All the same, by augmenting periodically the *vis a fronte* this would tend to bring more blood into the thorax. Mean intrathoracic pressure probably fell also (Eckstein & Hamilton, 1958) although this could not be deduced with certainty from records of intraoesophageal pressure alone.

If it was the increased inflow of blood into the thorax which caused polyuria in our experiments, the observed lack of quantitative correlation between changes in intrathoracic pressure variation and diuretic effect would appear to be attributable directly to the limited efficacy of the 'respiratory pump' (Brecher, 1956). It would also seem that the mechanism must be more sensitive than previous work has suggested. In the present experiments an increase in intrathoracic pressure variation of 25–30 mm H_2O or less, was accompanied by changes in urine flow entirely comparable in magnitude and time course to the polyuria described by workers who employed either continuous external negative pressures in the range -6 to -22 mm Hg, or rapid oscillations of ± 20 mm Hg in the pressure of the inspired air around a mean value set at various levels (Sieker *et al.* 1954; Surtshin, Hoeltzenbein & White, 1955; Love, Roddie, Rosensweig & Shanks, 1957; Murdaugh *et al.* 1959). In view of our finding that merely breathing through a low external airway resistance will alter the rate of urine flow it is possible that the polyuria observed by these investigators was also, at least in part, due to the resistance of valves and external airways, and only in part to superimposed pressure shifts or pulsations.

It has been suggested that recumbency diuresis itself is the result of a diminution in ADH secretion induced reflexly by the greater filling of the thoracic vasculature in the supine posture. In our experience the diuretic effect of altered breathing was more definite in recumbent subjects than in subjects who were seated, unless the latter had been given water to drink just before the experiment. It seemed that the mechanism concerned could

cause an appreciable loss of water from the body only after some preliminary lowering of ADH activity. This may be of importance in the evaluation of the hypothesis of Gauer & Henry (1956), who have proposed that reflexes arising in the chest—and accidentally activated by negative-pressure breathing—play a part in the normal regulation of body fluid volume.

SUMMARY

1. It has been shown that voluntary forced breathing, and breathing through external non-elastic airway resistances lead to a rise of urine flow. It was confirmed that acute anoxic anoxia, CO_2 inhalation, and negative-pressure breathing will do likewise.

2. All experimental modifications of breathing studied were equally effective in increasing water excretion. Polyuria did not depend on alterations of the volume of ventilation, tidal volume, rate of breathing, or on chemical changes in the blood.

3. A factor common to all procedures listed was augmentation of the respiratory fluctuation of intrathoracic pressure.

4. Such changes in sodium or total solute excretion, or in urine pH, as were observed were not statistically significant and not consistently related to increases in urine flow.

5. The findings were consistent with the hypothesis that during experimental modifications of breathing the secretion of antidiuretic hormone may be reduced by a reflex starting in intrathoracic deformation receptors, which are stimulated by increased filling of some portion of the central vascular bed when the respiratory intrathoracic pressure variation is increased.

We wish to thank our subjects for their co-operation, Professor G. M. Bull for a stimulating suggestion, and Dr M. I. M. Noble and Miss Ethel Cane for technical assistance. A grant from the Central Research Fund of the University of London to one of us (E. U.) for the purchase of apparatus used in this work is gratefully acknowledged.

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THE TRANSFER OF PROTEINS AND LIPIDS FROM PLASMA TO LYMPH IN THE LEG OF THE NORMAL AND HYPERCHOLESTEROLAEMIC RABBIT

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Experimental evidence concerning the exchange of materials between the circulating plasma and the tissue fluid supports the view that in the capillary wall there are pores through which diffusion of the small molecules and ions is relatively free, whereas diffusion of larger molecules is restricted (cf. Pappenheimer, 1953; Yoffey & Courtice, 1956). Pappenheimer postulated that pores of 60–90 Å in diameter in the capillaries of the preparation that he used, the mammalian hind leg, would account for the observed rates of transfer across the capillary wall of water and of lipid-insoluble molecules of various sizes. The position of these pores has not been clearly defined, although Chambers & Zweifach (1947) gave evidence which suggested that they were confined to the intercellular substance. Pappenheimer's experiments supported this view in that the total area of the pores was such that they could be limited to this region.

This concept of capillary permeability has been challenged in recent years by several workers using the electron microscope to determine the fine structure of the vascular endothelium. Bennett, Luft & Hampton (1959) have classified capillaries, according to the structure of the endothelium, into three main classes as follows: first, those in the liver and spleen, where there are intercellular gaps up to several thousand Å across, secondly, those in such tissues as the small intestine, kidney, parathyroid and hypophysis, in which intracellular fenestrations of 300–600 Å in diameter have been observed, and finally those in which there are no intercellular or intracellular pores. The third class is found in cardiac and skeletal muscle, lung, nervous system and skin, and in the endothelial cells of these capillaries there are numerous vesicles of endoplasmic reticulum, 200–600 Å in diameter. The basement membranes lining these different types of capillaries may also vary considerably in thickness and differentially affect the passage of materials from plasma to lymph. These findings in general confirm the work of others, e.g. Palade (1953, 1956), Fawcett (1955), Pease (1955), and have given rise to further controversy

concerning the mechanisms of transfer of materials across the capillary membrane.

Interest has also recently been aroused in the passage of various lipid complexes through the vascular endothelium, for it is thought that this phenomenon is concerned in the deposition of lipids in the intima of arteries in atherosclerosis. Electron microscopy of this endothelium has revealed the absence of pores or fenestrations, but the presence in the endothelial cells of numerous vesicles of cytoplasmic membrane similar to those observed in the cells of capillary endothelium (Buck, 1958).

The lipid complexes of the plasma are all much larger than the pores postulated by Pappenheimer. They vary in size from 150 Å, the probable size of the high-density α-lipoprotein (Hayes & Hewitt, 1957) to 5000 Å or more, the size of some chylomicrons, and are stabilized in an aqueous medium by the hydrophilic properties of phospholipid and protein.

Experiments have shown that these complexes may pass through the capillary membrane (Kellner, 1954; Courtice & Morris, 1955; Morris & Courtice, 1956) and that the ease with which they are transferred may in some way be related to their size (Courtice, 1959*a*, *b*). In these latter experiments, it was shown that whereas the lipid complexes in the lipaemic plasma of the rabbit fed on cholesterol or given Triton WR-1339 intravenously appeared in the lymph of the paw, the larger lipid particles of chyle or of the artificial fat emulsion 'Lipomul' (Upjohn) given intravenously did not do so to any significant extent.

The present investigations are a continuation of these experiments. They have been devised to show whether, in the hypercholesterolaemic rabbit, the passage of the various lipoprotein complexes across the membrane of the skin capillaries is related to the size of the complex concerned.

METHODS

Hutch-bred rabbits of various strains were used as experimental animals. Hypercholesterolaemia was produced by feeding the rabbits on pollard (bran-type flour from wheat) with which had been mixed powdered cholesterol in a blender. Each rabbit was given daily 75 g of pollard containing 1 g of cholesterol for the first week, and thereafter the cholesterol level was usually maintained at a level of 500–1000 mg/100 ml. by giving either this mixture or the pollard without cholesterol; green vegetables were also given several times a week.

To determine the plasma–lymph gradients across the wall of the skin capillaries, the animals were anaesthetized with pentobarbital (Abbott's Veterinary Nembutal) intravenously. Blood samples were taken from a catheter in the carotid artery and lymph samples from a lymphatic vessel just below the popliteal fossa (Courtice, 1959*a*). When a sample of lymph had been collected over a period of approximately 1 hr, the paw was injured by immersion in water at 70° C for 1 min. Before this procedure the animal was very deeply anaesthetized with ether. Two samples of lymph were then collected during the subsequent hour. The paw was kept in a water-bath at a constant temperature of 43–45° C throughout lymph collection, so as to ensure a fairly uniform blood flow. Lymph flow was promoted by passive movement of the leg and massage along the pathway of the duct.

Analytical methods. Total protein in plasma and lymph was determined by micro-Kjeldahl digestion, distillation and Nesslerization. The individual proteins were determined by paper electrophoresis on Whatman No. 1 paper, elution and determination of the dye, and measurement of the areas of the dye curves with a planimeter. As the globulins bind less dye than albumin, the areas of the dye curves for globulins were multiplied by the following factors: γ-globulin plus fibrinogen, 1·76; β-globulin, 1·40; and α-globulin, 1·50, before the protein concentrations were calculated. The detailed procedure adopted for these determinations is described elsewhere (Courtice, 1960). Total cholesterol in plasma and lymph was determined by the method of Abell, Levy, Brodie & Kendall (1952) and phospholipid by the method of Zilversmit & Davis (1950).

RESULTS

The lipid complexes in the plasma of normal and hypercholesterolaemic rabbits

In the normal rabbit fed on the pellets used in this laboratory, the cholesterol and phospholipid levels in the plasma were on an average 60 and 143 mg/100 ml. respectively and somewhat higher, 162 and 174 mg/100 ml., when fed on pollard. Havel, Eder & Bragdon (1955) by means of the preparative ultracentrifuge showed that these lipids were divided mainly between the high-density or α-lipoprotein and the lipoproteins within the density range 1·019–1·063 or β-lipoprotein. Hayes & Hewitt (1957) determined the size of these lipoproteins in human plasma by the electron microscope and found the diameter of the hydrated molecule to be 150 Å for the α-lipoprotein and 350 Å for the β-lipoprotein.

With increased cholesterol levels in the plasma of the cholesterol-fed rabbit, Gofman, Lindgren, Elliott, Mantz, Hewitt, Strisower, Herring & Lyon (1950) showed by the analytical ultracentrifuge that while there was a moderate increase in the concentration of these smaller lipoproteins, the main increase was in less dense complexes which dispersed light to make the plasma appear opalescent or milky. Under dark-ground illumination of the light microscope, very small particles up to approximately 0·2 μ (2000 Å) in diameter can be seen. The range in size of these lipoproteins is not known at present, but preliminary investigations by electron microscopy show that in such plasma the variation is from 150 Å to 1500 Å, with some larger particles.

In composition these lipid complexes in the plasma of the hypercholesterolaemic rabbit consist mainly of cholesterol and cholesterol esters stabilized with phospholipid and protein, and the density varies directly with the proportion of protein to lipid. In the lipid portion of the molecule or complex the ratio of cholesterol to phospholipid varies inversely with the density. In the high-density or α-lipoprotein fraction this ratio is on an average 0·4–0·5; as the complexes become less dense, it increases considerably to reach values of 3·0 or even higher. The relation between the cholesterol:phospholipid ratio and the total cholesterol in the plasma is

shown in Fig. 1. When the total cholesterol in the plasma was less than 100 mg/100 ml. the cholesterol:phospholipid ratio was on the average 0·49 and the lipid was mainly in the high-density lipoprotein. With levels between 100 and 200 mg/100 ml. the ratio rose to a mean of 0·93 and more of the lipid was present as β-lipoprotein, as determined qualitatively by zone electrophoresis. Above this level the ratio rose more gradually to values of 3·00 and the lipid was present mainly as the low-density complexes which gave the plasma a milky appearance. While the actual size of the

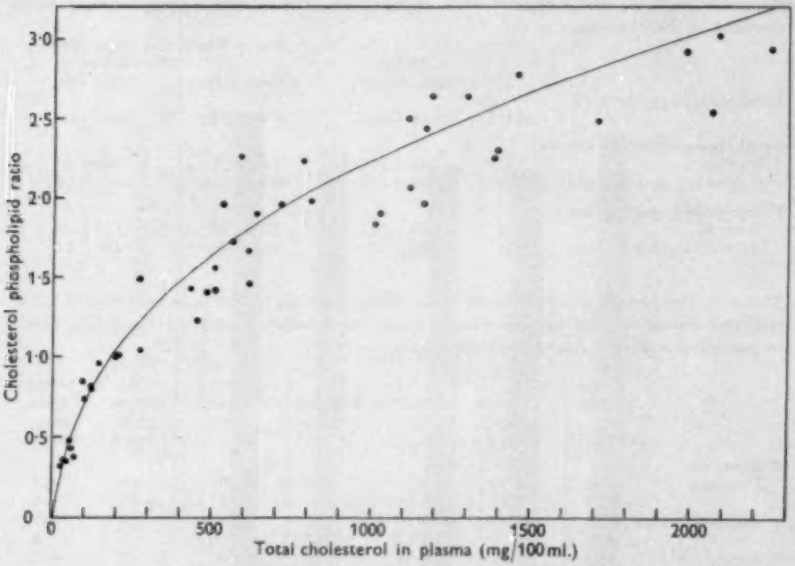

Fig. 1. The relationship between the level of cholesterol and the cholesterol: phospholipid ratio in the plasma of normal and hypercholesterolaemic rabbits.

different lipoproteins in the plasma has not yet been accurately determined, preliminary experiments suggest that this runs roughly parallel with the cholesterol:phospholipid ratio.

The plasma–leg lymph gradients of protein and lipids in normal and hypercholesterolaemic rabbits, before and after thermal burn to leg

In order to determine the protein and lipid gradients from plasma to lymph it was necessary to ascertain whether any changes in the composition of the plasma occurred while the lymph was being collected. Blood samples were obtained before Nembutal anaesthesia (from ear vein) and under anaesthesia (from the carotid artery) while lymph was being

collected, both before injury and at the end of the experiment after injury. The average figures, given in Table 1, showed a slight fall in the total plasma proteins throughout the experiment and a slight rise in cholesterol and phospholipid. In relating the levels in the lymph to those in the plasma, the figures for the plasma during anaesthesia before injury were used. Any changes in the levels in the plasma would be reflected in the

TABLE 1. Total protein, cholesterol and phospholipid concentration in plasma taken before Nembutal anaesthesia and during anaesthesia before and after thermal injury to the paw. Expressed as mean and standard error of the mean. The figures in parentheses give the numbers of observations

		Before anaesthesia	Under Nembutal anaesthesia	
			Before injury	After injury
Total protein (g/100 ml.)	(14)	6·86 ± 0·17	6·70 ± 0·16	6·47 ± 0·18
Total cholesterol (mg/100 ml.)				
Normal	(9)	143 ± 16	143 ± 17	140 ± 20
Hypercholesterolaemic	(8)	929 ± 214	1005 ± 224	1022 ± 213
Phospholipid (mg/100 ml.)				
Normal	(9)	168 ± 12	154 ± 12	153 ± 13
Hypercholesterolaemic	(8)	422 ± 55	446 ± 61	454 ± 44

TABLE 2. The protein and lipid levels in the plasma and leg lymph of normal and of hypercholesterolaemic rabbits, expressed as the mean and standard error of the mean. The figures in parentheses give the number of observations

	Total protein (g/100 ml.)	Albumin (g/100 ml.)	α-globulin (g/100 ml.)	β-globulin (g/100 ml.)	γ-globulin and fibrinogen (g/100 ml.)	Total cholesterol (mg/100 ml.)	Phospholipid (mg/100 ml.)
Normal (6)							
Plasma	6·76 ± 0·18	3·70 ± 0·11	0·72 ± 0·05	0·96 ± 0·08	1·37 ± 0·17	149 ± 22	143 ± 16
Lymph, before injury	2·81 ± 0·30	1·75 ± 0·16	0·28 ± 0·03	0·39 ± 0·05	0·40 ± 0·09	35 ± 6	47 ± 4
Lymph, after injury	5·54 ± 0·21	3·14 ± 0·16	0·56 ± 0·06	0·80 ± 0·05	1·04 ± 0·15	90 ± 16	106 ± 12
Hypercholesterolaemic (9)							
Plasma	6·66 ± 0·25	3·49 ± 0·23	0·70 ± 0·06	1·19 ± 0·08	1·28 ± 0·17	798 ± 117	407 ± 36
Lymph, before injury	2·78 ± 0·26	1·68 ± 0·25	0·27 ± 0·03	0·36 ± 0·04	0·47 ± 0·06	98 ± 11	83 ± 10
Lymph, after injury	4·97 ± 0·34	2·71 ± 0·23	0·47 ± 0·05	0·77 ± 0·06	1·02 ± 0·13	371 ± 53	209 ± 15

values for lymph after a lag for mixing in the tissue-fluid pool. This mixing would be relatively slow before injury and rapid after injury. As there is little difference in the levels of protein and lipids in the two samples taken during anaesthesia, it was thought that these levels should be used for comparison with the values for lymph.

While the plasma–lymph gradients for the lipids were determined in a larger series, the individual plasma proteins, together with the lipid levels

were measured in six normal and nine hypercholesterolaemic animals. The results are given in Table 2. The mean levels of each individual protein in the plasma and in the lymph before injury were approximately the same in both groups of animals. In each group the blood pressure, determined by the capsule method in the ear artery, was approximately the same, and the limb was kept at a constant temperature while lymph was collected. The fact that the plasma–lymph gradients for the different proteins were approximately the same in both groups under these conditions would

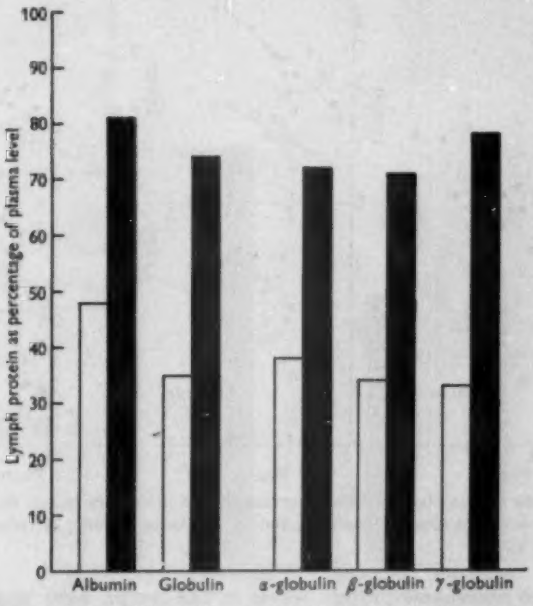

Fig. 2. The mean levels of the various protein fractions in the lymph from the leg, expressed as percentages of the corresponding levels in the plasma of a group of 15 rabbits. □, before injury; ■, after thermal injury to leg.

suggest that there was no appreciable change in permeability of the skin capillaries to the plasma proteins due to the increased lipid levels. When the leg was injured by a thermal burn, however, gross oedema rapidly developed and the lymph flow increased greatly (cf. Courtice, 1946). The concentrations of each of the protein fractions and of the lipids in the lymph rose considerably.

When expressed as a percentage of the plasma levels, the concentrations of the proteins in the lymph were as shown in Fig. 2. In lymph from the leg before injury the plasma–lymph gradient for albumin was less than for

total globulin or for any of the individual globulins. The mean concentration of albumin in the lymph in these experiments was 48 % while that of globulin was 35 % of the corresponding levels in the plasma. The relative levels of each of the individual globulin fractions did not vary appreciably from that of the mean total globulin. In lymph after injury the difference in the gradients for albumin and globulin was less marked, but still evident. The mean concentration of albumin in the lymph was 81 % while that of the total globulin was 74 % of the corresponding levels in the plasma.

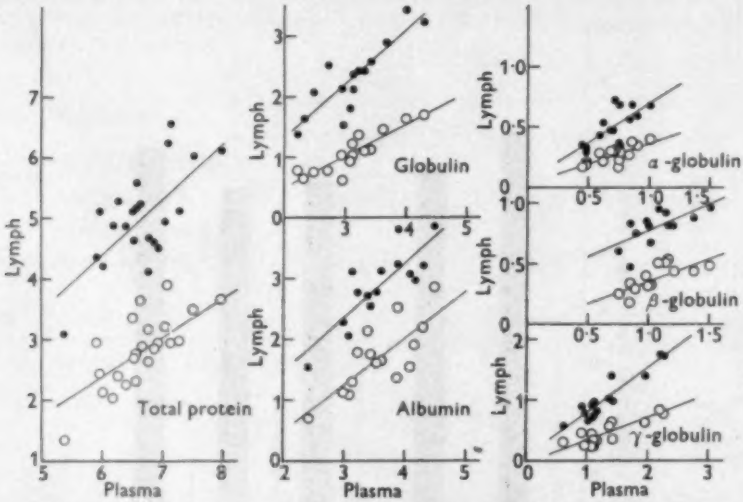

Fig. 3. The relation between the concentration of protein in lymph from the leg and that in the plasma; figures are g/100 ml. ○, before injury; ●, after thermal injury to leg.

When the individual protein levels in the lymph were plotted against those in the plasma, the regression equations showed that the concentrations in the lymph were related to those in the plasma, $P < 0{\cdot}001$ (Fig. 3). The slope of the regression line for albumin was steeper than those for the globulins, $P < 0{\cdot}05$. After injury the slope of the regression line for globulin increased to show no significant difference from that for albumin. These findings are all in conformity with the classical 'Pore Theory' of capillary permeability.

With regard to the lipid complexes, in the normal animal the levels of cholesterol and phospholipid in the lymph relative to those in the plasma were slightly less than in the α- and β-globulin (Fig. 4). In this series the levels of cholesterol and phospholipid in the lymph were respectively 24 and 33 % of the plasma levels before injury and 60 and 74 % after injury.

This is probably due to the fact that α- and β-lipoprotein molecules are larger than other globulins that migrate electrophoretically at the same rate. The finding that the relative levels of cholesterol and phospholipid varied also shows that in the normal animal there must be different plasma–lymph gradients for α- and β-lipoproteins.

In hypercholesterolaemic animals the levels of cholesterol and phospholipid in the lymph relative to those in the plasma were lower than in normal animals, i.e. the plasma–lymph gradients were greater. The average for

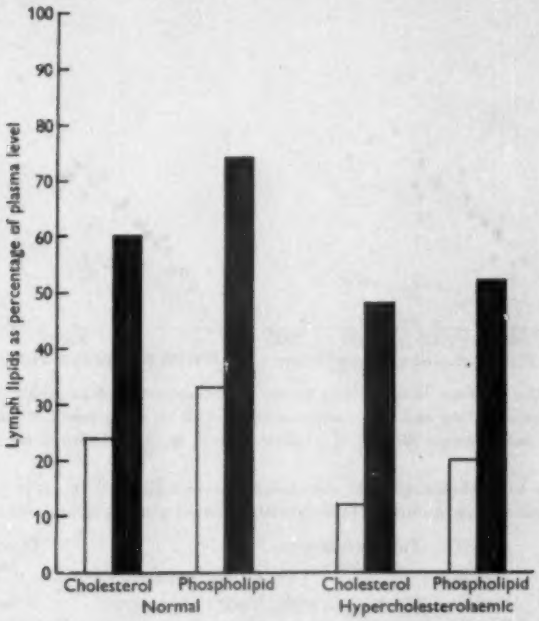

Fig. 4. The mean levels of cholesterol and phospholipid in the lymph from the leg, expressed as percentages of the corresponding levels in the plasma in a group of 6 normal and 9 hypercholesterolaemic rabbits. □, before injury; ■, after thermal injury to leg.

the experiments represented in Fig. 4 shows that the cholesterol and phospholipid levels in the lymph were 12 and 20% of the plasma levels respectively before injury and 48 and 52% after injury. These differences again show the differential filtration of the various lipid complexes in hypercholesterolaemic plasma.

In a larger series of animals in which plasma and lymph lipids have been measured, the results are depicted in Fig. 5. This shows that when the plasma cholesterol increases from 40 to 200 mg/100 ml., the lymph

cholesterol rose much more steeply than with changes in the plasma level from 500 to 2200 mg/100 ml. The changes in plasma–lymph gradient with rising plasma cholesterol are more clearly depicted in Table 3. Fourteen animals on a diet to which no cholesterol was added have been divided into two groups, with ranges of plasma cholesterol of 0–100 and 101–200 mg/100 ml. Similarly twenty-two animals on a diet to which cholesterol was

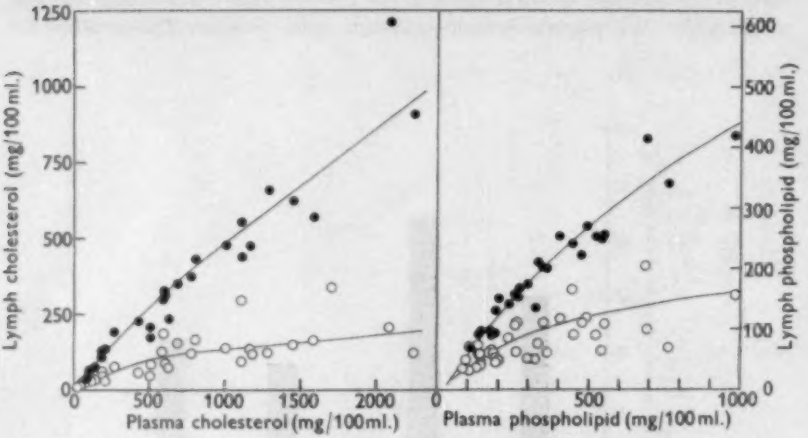

Fig. 5. The relation between the levels of cholesterol and phospholipid in the lymph from the leg and the corresponding levels in the plasma of normal and hypercholesterolaemic rabbits. ○, before injury; ●, after thermal injury to leg.

Table 3. The total cholesterol and the cholesterol:phospholipid ratios in plasma and leg lymph in four groups of rabbits with different plasma cholesterol levels

Plasma cholesterol range (mg/100 ml.)	No. of animals	Total cholesterol (mg/100 ml.)		Lymph cholesterol / Plasma cholesterol × 100	Cholesterol:phospholipid ratio	
		Plasma	Lymph		Plasma	Lymph
0–100	8	60 ± 9	23 ± 5	38 ± 4	0·49 ± 0·07	0·42 ± 0·06
101–200	6	162 ± 18	37 ± 5	23 ± 2	0·93 ± 0·04	0·77 ± 0·12
201–1000	11	585 ± 46	108 ± 14	18 ± 2	2·02 ± 0·17	1·29 ± 0·10
1001–2000	11	1450 ± 126	171 ± 24	12 ± 2	2·45 ± 0·13	1·47 ± 0·10

added have been divided into two groups, with ranges of plasma cholesterol of 201–1000 and 1001–2000 mg/100 ml. In the first group, with a mean plasma cholesterol of 60 mg/100 ml., most of the cholesterol is present as α-lipoprotein. The level of cholesterol in the lymph was 38 % of the plasma level and the cholesterol:phospholipid ratio was nearly the same as that in the plasma. As the level of cholesterol in the plasma rose, the relative

concentration in the lymph fell and the difference between the cholesterol:phospholipid ratios of plasma and lymph became progressively greater.

When the skin capillaries were injured by heat, however, larger lipid complexes more readily passed into the lymph, with much less differentiation according to the size of the complexes (Fig. 5). The lymph, which was opalescent before injury, became quite milky in appearance. It would

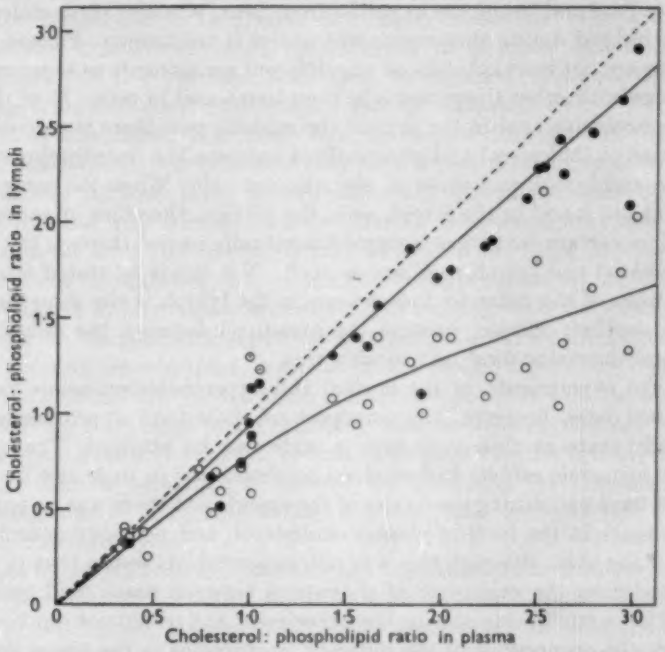

Fig. 6. The relation between the cholesterol: phospholipid ratio in the lymph to that in the plasma in normal and hypercholesterolaemic rabbits. The interrupted 45° line represents identity of ratios. ○, before injury; ●, after thermal injury to leg.

seem, therefore, that in the uninjured capillaries there is a considerable differentiation in the plasma–lymph gradients between the various lipid complexes, while in the injured capillaries this differentiation, though still detectable, is much less.

These conclusions can also be drawn when the cholesterol:phospholipid ratio in the lymph is plotted against the cholesterol:phospholipid ratio in the plasma (Fig. 6). In the uninjured limb the ratio in the lymph was approximately the same as in the plasma at levels below 0·5, when

most of the lipids were present as α-lipoprotein. As the plasma ratio rose, the lymph ratio rose less steeply, indicating that the larger, less dense complexes enter the lymph less readily than the smaller molecules. In the injured limb, however, this differentiation was much less marked, but still evident to some slight extent.

DISCUSSION

The mechanisms concerned in the transfer of macromolecules from plasma to tissue fluid and lymph are as yet far from clear. Whether these molecules are unchanged during the process of transfer is not known. Plasma lipoproteins are not inert substances; the different components to some extent exchange with other lipoproteins in the plasma and in cells. It is, therefore, conceivable that in the skin of the rabbit's paw there is a continual exchange of cholesterol and phospholipid between the lipoproteins in the plasma and lymph and those of the adjacent cells. When the same lipoproteins are found in the lymph as in the plasma, therefore, it cannot be stated for certain that these lipoproteins actually passed through the walls of the blood and lymph capillary as such. Nor can it be stated that the proportion of the different lipoproteins in the lymph is the same as that in the capillary filtrate, because the exchanges between the cells in the skin and the tissue fluid may modify this.

In the experiments in the normal and hypercholesterolaemic rabbits described here, however, the transport of cholesterol approximated to a steady state as closely as such a state can be attained. The hypercholesterolaemic rabbits had received no cholesterol in their diet for from 2 to 14 days and during the course of the experiment there was no appreciable change in the level of plasma cholesterol, and probably none in the cells of the skin, although this was not measured. It seems that in these circumstances the exchanges of cholesterol between tissue fluid and cells would be in equilibrium during the experiment and would not significantly modify the proportions of the different lipoproteins in the tissue fluid.

If this is so, the levels of the various proteins and lipoproteins in the tissue fluid, though actually higher than in capillary filtrate, would be in the same relative proportions, since these molecules do not diffuse back into the blood stream. As lymph has the same composition as tissue fluid from any region (Yoffey & Courtice, 1956), the plasma–lymph gradients of the proteins and lipoproteins will, therefore, give a relative measure of the plasma–capillary filtrate gradients.

The great increase in the rate of transfer of the various proteins and lipoproteins from plasma to lymph when the skin is injured by a thermal burn suggests that this is mainly a transfer of the complexes as they exist in the plasma. Within seconds after injury the lymph may become more

opalescent. The sudden change in the composition of the lymph and the large bulk transfer of plasma, together with the finding that the gradients for proteins and lipoproteins vary with the size of the molecule, support this view. The results show clearly that the plasma–lymph gradient progressively increases from the smallest protein molecule, albumin, to the largest lipoprotein complex found in the plasma. The experiments of Courtice (1959*a*, *b*) show further that lipid complexes which are even larger, such as the particles in chyle or the artificial fat emulsion 'Lipomul', are not transferred from plasma to lymph, even after injury, to any measurable extent. Although the diameter of all these molecules and complexes has not yet been accurately determined, it seems that the plasma–lymph gradient runs roughly parallel with their size.

If it is true, then, that these complexes pass through the capillary membrane as such, it is clear that they cannot pass through pores 60–90 Å in diameter without being grossly distorted. Electron microscopy has failed to show any fenestrations or pores in the intercellular substance except in the liver and spleen. It may be that in the living capillary the intercellular substance is in a continually changing state, as the pressure on capillaries in any region is continually changing with vasomotion. If this were so, the intercellular substance might at times be pulled somewhat apart causing fenestrations, whereas in the fixed preparation observed with the electron microscope there is a shrinkage. It is well known that in the living state large blood cells may pass between endothelial cells if they stick on the endothelium, so that large gaps may for a time be produced. Until a technique is devised for obtaining the requisite high magnifications in living capillaries, however, this question will not be settled conclusively.

If the lipoproteins do not pass through the intercellular substance, they must pass through the cells. Here the vesicles of endoplasmic reticulum may be implicated. Aksne (1959) has shown that the endothelial cell layer of the capillaries in the skin of the mouse varies in thickness from 4·0 to 0·25 μ, being thickest in the part of the cell containing the nucleus. Particles of colloidal mercuric sulphide 50–250 Å in diameter introduced into the blood-stream passed through the cytoplasm and were found within intracellular membrane structures such as the vesicles. When the capillaries were injured by histamine, there was still no evidence of pores in the intercellular substance but the vesicles increased in size, some becoming as large as 2000 Å in diameter, giving the cells a foamy or vesicular appearance. Aksne postulated an active transfer across the endothelial cells; he envisaged engulfment of material on the luminal side through the 'pinching off' of membrane-lined caveolae, the vesicles so formed being then conveyed across the endothelial cells to discharge their contents into the

extracellular space outside the endothelium. The basement membrane then forms a further barrier which affects the further transfer of any substance.

The sizes of the lipid complexes in the plasma of the hypercholesterolaemic rabbit are in conformity with the diameters of the vesicles observed in the endothelial cells of skin capillaries of the mouse before and after histamine application. The capillaries in the skin of the rabbit's paw before and after thermal injury have not yet been similarly investigated, but if the vesicles change in size, as in the mouse, it is conceivable that they might be implicated in the transfer of the lipid complexes. No one has yet reported, however, the finding of such lipid particles in these vesicles. In the endothelium of the aorta, over a plaque in the hypercholesterolaemic rabbit, Poole & Florey (1958) have shown lipid in the cytoplasm of the cell. Whether this was in the vesicles was not determined. Hence, although the experimental results suggest that the lipid complexes of the hypercholesterolaemic rabbit are filtered through a sieve-like arrangement, the mechanisms cannot as yet be clearly defined.

SUMMARY

1. The concentrations of the protein fractions, albumin and α-, β- and γ-globulins and also cholesterol and phospholipid were determined in the plasma and leg lymph of groups of rabbits fed on a normal diet and on a diet to which cholesterol was added before and after thermal injury to the leg.

2. The plasma–lymph gradients thus obtained showed that in the lymph from the uninjured leg the level of albumin was 48% and total globulin 35% of the corresponding concentrations in the plasma. The level of cholesterol in the lymph was on an average 38, 23, 18 and 12% of the plasma level respectively when the plasma cholesterol was within the ranges 0–100, 101–200, 201–1000 and 1001–2000 mg/100 ml.

3. After injury the plasma–lymph gradients of all these substances were much less, and, while there was still a progressive change in the gradient from albumin to the largest lipid complex, this differentiation was less marked than in the uninjured leg.

4. The results indicated that the plasma–lymph gradient of the proteins and lipoproteins in normal and hypercholesterolaemic rabbits depended, to some extent at least, on the size of the molecule or complex concerned. Possible mechanisms of transfer of these macromolecules or complexes across the capillary wall are discussed.

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MEMBRANE CHANGES OF *ONCHIDIUM* NERVE CELL IN POTASSIUM-RICH MEDIA

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In previous work (Hagiwara & Saito, 1959*a*, *b*) the current–voltage relations of the membranes of several nerve cells were studied in normal bathing media. When the potassium concentration of the medium was increased a number of changes appeared in the current–voltage relations. Recently work has shown that in potassium-rich media threshold phenomena are present both in the nodal membrane of the myelinated fibre (Mueller, 1958*a*, *b*; Stämpfli, 1958; Tasaki, 1959) and in the membrane of the squid axon (Segal, 1958; Moore, 1959; Tasaki, 1959). In the present paper similar phenomena have been investigated in the nerve cell of *Onchidium verruculatum* immersed in potassium-rich media. The changes were also analysed in the presence of narcotics.

METHODS

The sub- and supra-oesophageal ganglia of a marine pulmonate mollusc, *Onchidium verruculatum*, were used. The technique of preparation was similar to that employed previously (Hagiwara & Saito, 1959*b*). The preparation was fixed on the bottom of a Lucite box (0·5 × 5 × 3 cm). Experimental solutions flowed into the box through a plastic tube at one end and drained at the other end. By this procedure the external solution could be changed while the tip of an electrode remained inside the cell.

Glass capillary tubes of tip diameter less than 0·5 μ, filled with 3 M-KCl, were used for recording the membrane potential change as well as for polarizing the cell membrane. The indifferent electrode was an agar-sea-water-3 M-KCl-AgCl-Ag electrode immersed in the external solution. Sometimes agar in KCl sea water was used instead of agar in normal sea water. To measure the resting potential while the external solution was changed, another micro-electrode was placed just outside the impaled cell and the membrane potential was recorded as the potential difference between the two micro-electrodes by using a differential cathode-follower system. The cathode follower and feed-back system for the voltage-clamp experiments were similar to those described previously (Hagiwara & Saito, 1959*a*).

The artificial sea water had the following composition (mM): NaCl, 465; KCl, 9·3; $CaCl_2$, 10·8; $MgCl_2$, 48·3; $NaHCO_3$, 5·9; pH, 8·1. The KCl sea water was similar to normal sea water except that NaCl was replaced by KCl of equivalent molarity. In order to remove chloride from the solution the chloride of normal sea water was replaced by sulphate, and the composition of the sulphate sea water was (mM): Na_2SO_4, 233; K_2SO_4, 48·3; sucrose, 315. $CaSO_4$ was added

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to saturation. Sucrose was added to maintain the isotonicity of the solution. Replacement of Na with K, or of Cl with SO_4, was performed to any desired extent by mixing appropriate amounts of these three solutions.

RESULTS

After-potential and current–voltage relations of the normal cell membrane

When a micro-electrode was inserted into a cell immersed in normal sea water a resting potential which ranged between 45 and 60 mV was recorded. A spike potential appeared when an outward current pulse decreased the resting potential by about 20 mV. A positive after-potential or undershoot followed the spike and the membrane potential at the peak of the undershoot was 8–18 mV more negative than the resting potential. The decrease of resting potential by a subthreshold outward constant-current pulse followed a simple exponential time course when applied to the resting membrane (Fig. 1*A*). This indicates that no secondary change of the membrane was associated with a subthreshold potential change. However, secondary change was seen when an outward current pulse was applied to the membrane hyperpolarized by a conditioning inward current. As is shown by Fig. 1*B*, the rate of potential rise suddenly became slower when the membrane potential reached a level near to the peak potential level of the undershoot, and the potential tended to stay at this level. Since this potential change appeared at the break of the inward current it will be called the 'break response' in the following description.

When the membrane potential of the cell was suddenly shifted to various potentials by the voltage-clamp technique the associated membrane current finally reached a steady intensity after a certain transient change (Hagiwara & Saito, 1959*b*). The current–voltage relation of the membrane at the steady state (1 sec after the start of the voltage change) is shown in Fig. 1*E* by filled circles. In this figure the abscissa indicates the membrane potential measured from the resting level and the ordinate shows the absolute value of the membrane current, the depolarization and outward current being taken as positive. The relation was linear for hyperpolarization as well as for small depolarizations but a marked deviation from the straight line was found for membrane potential changes larger than about 25 mV. This corresponds to the delayed rectification of the cell membrane (Hagiwara & Saito, 1959*b*). The two-step voltage-clamp technique was employed to examine further the current–voltage relation of the cell membrane during the break response. The membrane potential was first clamped to a conditioning level of hyperpolarization, and after the membrane current had reached a steady state the potential was changed to a second level. As is shown by Fig. 1*C*, an initial rapid outward surge appeared immediately after the change of the membrane potential from the conditioning level to

the test level when the latter reached a certain critical value. This initial outward surge presumably corresponded to the break response in the absence of voltage clamp. When the test step was subthreshold for the initial surge, it produced a capacitative current followed by a constant current.

The current–voltage relation at the peak of the surge is illustrated in Fig. 1*E* by open circles. In this case the abscissa indicates the difference between the membrane potential at the test step and the resting potential level and the ordinate shows the absolute value of the membrane current at the peak of the surge. The relation deviated from the steady-state rela-

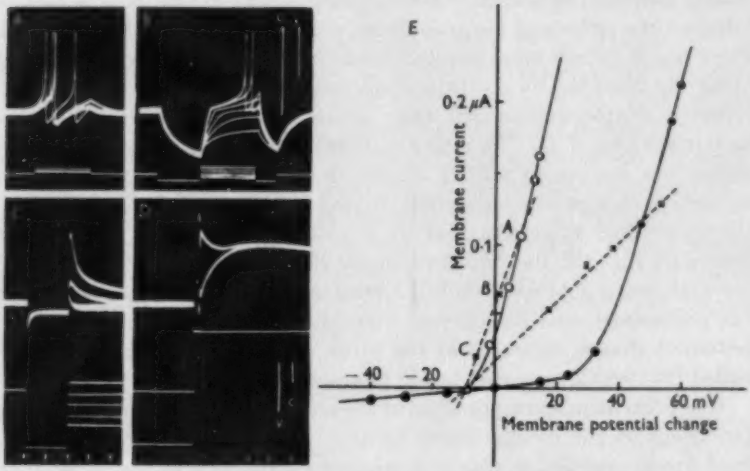

Fig. 1. Potential changes and membrane currents of cells immersed in normal sea water. *A* and *B*, potential changes (upper trace in each record) produced by outward current pulses with and without a conditioning inward current (shown in lower trace). *C*, membrane currents (upper trace) associated with a sudden shift of the membrane potential (lower trace) to various levels from a certain hyperpolarization level. *D*, membrane currents (upper trace) associated with a rectangular depolarization (lower trace). Two recordings were superposed, in one of which hyperpolarization preceded the depolarization. The preceding hyperpolarization induced an initial outward surge. Upward direction of current trace indicates outward direction of membrane current. Calibrations in *A*, *B* and *C*: *v*, 50 mV; *c*, 0·2 μA; time marker, 100 msec. Calibration in *D*: *v*, 50 mV; *c*, 0·2 μA; time marker, 20 msec. *E*, current–voltage relations of the cell membrane in normal sea water. Membrane potential measured from the resting potential level was plotted on the abscissa, and amplitude of membrane current on the ordinate. Positive-going potential change or outward direction of current, positive on each axis. Filled circles give the relation at the steady state of a single-step voltage clamp, and open circles that at the peak of the initial surge obtained by a two-step voltage clamp when the conditioning hyperpolarization was 70 mV. Interrupted lines show the method for measuring equilibrium potentials at the steady state and at the peak of the initial surge.

tion for the membrane potential changes larger than −10 mV. After the initial surge or after a subthreshold change the membrane current reached a steady state determined by the membrane potential at the test step. The current–voltage relation at the steady state was similar to that obtained by the single-step voltage-clamp technique.

In order to estimate the membrane conductance and the membrane equilibrium potential at the peak of the outward surge, the clamped membrane potential was further changed to various third levels just after the peak of the surge had appeared and the current immediately after the third potential change was estimated after eliminating an appropriate capacitative current. The points *B* and *C* were obtained by plotting the current intensity against the membrane potential at the third step when the intensity of the outward surge and the potential level at the second step were given by point *A*. The three points lie approximately on a single straight line and the conductance determined from the slope was about 25 times larger than the resting membrane conductance. The membrane equilibrium potential estimated from the intercept on the potential axis was about −10 mV. Although a slightly different conductance was obtained for a different value of the membrane potential at the second step, a common value was always found for the equilibrium potential. The results, therefore, indicate that the break response is produced by a transient conductance increase of the membrane with a certain equilibrium potential which is in this case about 10 mV more negative than the resting potential. Although the equilibrium potential showed a variation from cell to cell, it always coincided with the potential level at the peak of the undershoot.

In the previous paper (Hagiwara & Saito, 1959*b*) it was concluded that the slowly developing rectifying current is produced by a conductance increase of the membrane with a certain equilibrium potential which is also similar to the potential level at the peak of the undershoot. Therefore, this rectifying current and the current associated with the break response are produced by conductance increases with a common equilibrium potential. A straight line denoted by *a* in Fig. 1*E* was obtained by a two-step voltage-clamp technique, indicating the coincidence between these two equilibrium potentials.

Although the equilibrium potentials were common for these two kinds of conductance increases, there was apparently no interaction between them. Two recordings of membrane currents associated with rectangular depolarizations of the same amplitude were superposed in Fig. 1*D*; in the one the rectangular depolarization was preceded by a conditioning hyperpolarization and in the other not. An initial outward surge was superposed on the slowly developing rectifying current when the depolarization was preceded by the hyperpolarization, but this did not influence the final time course.

From the foregoing results it is concluded that the three membrane potentials, the equilibrium potential for the slowly developing rectifying current, that for the break response, and the potential level at the peak of the undershoot, all give the same value, and that it is 5–15 mV more negative than the resting potential when the cell is in the normal sea water containing 9·3 mM-K.

After-potential in potassium-rich media

When the cell was immersed in a medium in which part of the Na had been replaced by an equivalent of K, a characteristic change occurred in the after-potential. The series of records *A*, *B*, *C* and *D* in Fig. 2 were obtained when the applied solutions contained 55·8, 102·3, 164·3 and

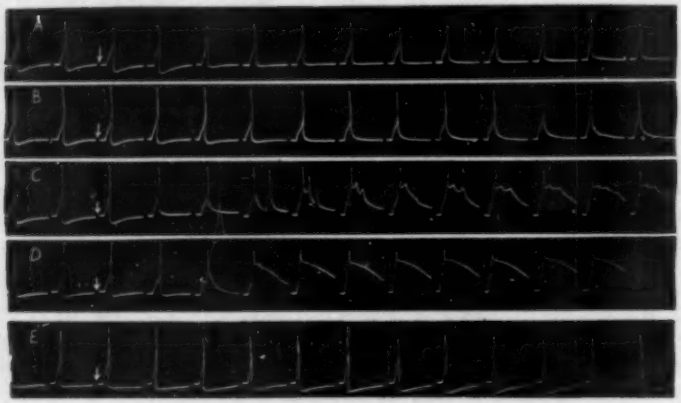

Fig. 2. Effects of changes in K concentration on the spike potential. In each series spike potentials were recorded at 5 sec intervals following the immersion of the cell in 55·8 mM-K (*A*), 102·3 mM-K (*B*), 164·3 mM-K (*C*), 474·3 mM-K (*D*) and K-free (*E*) sea waters. Cl concentration was normal in these solutions. The start of application of test solutions is indicated by arrows. Voltage calibration, 50 mV; time marker, 20 msec.

474·3 mM-K respectively, but the Cl concentration was kept normal. In every series the spike recordings were obtained at 5 sec intervals during the application of the solution. In each series an arrow indicates the start of application of the test solutions. The elevation of the after-potential became marked as the K concentration increased. At 164·3 mM-K (Fig. 2*C*) the membrane potential during the after-potential was above the firing level for the spike and, therefore, second and third spikes were initiated. Small oscillations superposed on the after-potential seen in the later spikes of this series were probably due to electrotonic spread of spike potentials

initiated in the axon some distance away from the cell soma. These spikes probably did not invade the soma because the latter was incapable of producing a spike potential during such an after-potential. When the K concentration was increased to 474·3 mM (all of the Na in the sea water was replaced by K, Fig. 2*D*) the after-potential was prolonged, and formed a plateau. The potential level at the plateau was about 40–50 mV more positive than the resting potential. Since the resting potential of the cell had a similar size this level corresponded approximately to zero membrane potential. Under such conditions the spike potential became very similar to that of cardiac muscle (Weidmann, 1951, 1956) and also of nerve cells treated with tetraethylammonium chloride (Hagiwara & Saito, 1959*b*). However, this similarity seemed to be only superficial, because the plateau was associated with a large increase of the membrane conductance, while a decrease or no increase was found during the plateau of the cardiac muscle or the tetraethylammonium-treated nerve cell (Weidmann, 1951; Hagiwara & Saito, 1959*b*). Figure 2*E* was obtained when the external potassium was removed. The potential level at the peak of the undershoot became more negative in the K-free solution. In elevated potassium the break of a sufficiently large inward current resulted in a potential change similar to the after-potential of the spike (Fig. 3*A*). This was considered to be a 'break response'. At some concentrations of K the break response was preceded by a spike but its amplitude was usually small. All these effects of potassium were completely reversible when the cell was brought back to the normal sea water.

The change of the after-potential reached a steady state about 40 sec after the change of the external solution had started. Since in the present experiments the test solution flowed slowly into the box in which the preparation was placed, the delay of 40 sec could be accounted for by the time for diffusion of the test solution around the outside of the cell. In contrast to the marked changes in the after-potential and break response no appreciable change was found in the resting potential within this period. The resting potential changed with a much slower time course, as is shown by Fig. 3*B*. In this part of the figure, record *B* 1 was obtained in normal sea water while the other five records were obtained 20 sec, 40 sec, 90 sec, 5 min and 8 min after the cell had been immersed in KCl sea water. In record *B* 4 a superimposed trace marked by an arrow shows the reference potential level, indicating that the resting potential had a small negative value and the membrane potential at the peak of the break response was slightly positive: the interrupted line in each record indicates the original resting potential level. The slow decrease of the resting potential could not be due to slow diffusion. In other words the results suggest that an increase of K concentration does not immediately cause a decrease of the resting

potential, and that a decrease is only produced after some secondary change has taken place in the cell, such as the change of concentration of some ion or ions inside the cell. In a few experiments the cell was brought back to the normal [K] medium after the resting potential had become steady. The recovery of the resting potential occurred with a very slow time course, while the peak level of the break response returned to normal within a much shorter time. This fact also suggests that the decrease of the resting potential is due to some secondary change.

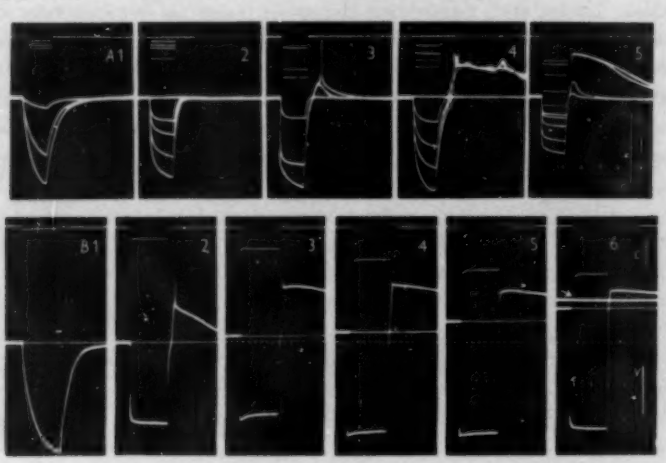

Fig. 3. *A*, membrane potential changes (lower traces) produced by inward current pulses (shown in upper traces) applied to cells immersed in normal (1), 55·8 mM-K (2), 102·3 mM-K (3), 164·3 mM-K (4) and 474·3 mM-K (5) sea waters. Cl concentration was normal in all cases. The recordings were taken before the decrease of resting potential. Calibrations: *v*, 50 mV; *c*, 0·2 μA; time marker, 100 msec. *B*, long-term time course of potential changes (lower traces) of the cell membrane produced by an inward current pulse (upper traces) following the immersion of the cell in KCl sea water (474·3 mM-K). Records 1–6 were obtained, respectively, in normal sea water, and about 20 sec, 40 sec, 90 sec, 5 min and 8 min after the immersion in KCl sea water. Calibrations: *v*, 50 mV; *c*, 0·4 μA; time marker, 100 msec.

When the K concentration was raised in such a manner that the product of K and Cl concentrations was kept constant the results were entirely different. The records in Fig. 4 were obtained when the Cl concentration was reduced by replacement with an equivalent amount of SO_4, [K] being increased to keep the product constant. The resting potential decreased with a time course similar to that observed for the change of the after-potential in the experiment shown in Fig. 2. Thus the elevation of the after-potential was not seen, since the resting potential and the peak of the after-potential changed simultaneously and both reached the same final

steady level which was determined by the concentration of external K. Recovery of the resting potential also occurred in a short time when the cell was returned to the normal sea water. This was in contrast to the manner in which the resting potential decreased in KCl media. The results indicate that the concentration gradient of Cl is one of the important factors which govern the size of the resting potential. However, it did not seem to be the only factor, since even the total replacement of the Cl in the sea water with SO_4 did not produce any appreciable change of the resting potential if the K concentration was kept normal. Therefore, the resting potential of the

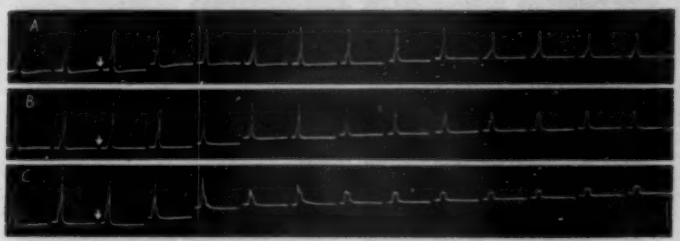

Fig. 4. Effects of increasing K concentration with simultaneous decrease of Cl concentration. Spike potentials in each series were obtained at 5 sec intervals following the application of: (*A*), 102·3 mM-K and 53·9 mM-Cl; (*B*) 164·3 mM-K and 33·5 mM-Cl; and (*C*), 474·3 mM-K and 11·2 mM-Cl sea waters. The start of application of test solutions is marked by arrows. Calibration, 50 mV; time marker, 100 msec.

present cell is concluded to be determined by the concentrations of K and Cl. Although there are some characteristic differences a similar phenomenon has been observed in muscle fibres by Hodgkin & Horowicz (1959). In contrast to this the peak potential level of the after-potential or the break response depended exclusively on the concentration of K.

Current–voltage relations of the cell membrane in K-rich media

The records in Fig. 5*A* and *B* were obtained with a cell immersed in a medium containing 102·3 mM-K (with normal Cl concentration) when the resting potential was still normal. An outward constant current pulse produced a spike potential when the depolarization reached about 20 mV, and the time course of the subthreshold depolarization followed a simple exponential time course as in normal sea water though the membrane showed a smaller time constant due to decrease of the resting membrane resistance. When an outward current pulse was superimposed on a conditioning inward current a marked deviation appeared in the time course of the potential change at a certain membrane potential and the deviation was in this case upward (Fig. 5*A*, *B*).

Corresponding changes were found in the current–voltage relations when observed by voltage-clamp technique. In Fig. 5*C* the steady-state relation is given by filled circles while the relation at the peak of the initial surge with a conditioning hyperpolarization is shown by open circles. The initial surge appeared at about -10 mV and this potential level was similar to that observed for the initial surge before the increase of K concentration. However, the direction of the surge was inward at this potential level. The amplitude of the inward surge showed a small increase as the membrane potential changed from -10 mV to $+10$ mV, but with further positive

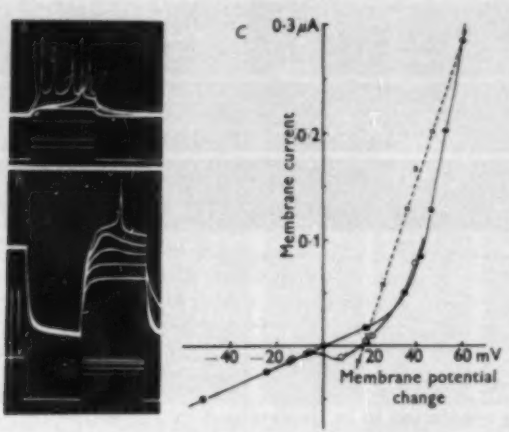

Fig. 5. *A* and *B*, potential changes (upper traces) produced by outward current pulses (shown in lower traces) applied to the resting (*A*) and hyperpolarized (*B*) cells in 102·3 mM-K and normal [Cl] sea water. The records were obtained before the decrease of resting potential. Calibrations: *v*, 50 mV; *c*, 0·2 μA; time marker, 100 msec. *C*, current–voltage relations of cell membrane in 102·3 mM-K and normal [Cl] sea water. Filled circles give the relation at the steady state of a single-step voltage clamp, and open circles that at the peak of the initial surge obtained by a two-step voltage clamp when the conditioning hyperpolarization was 60 mV. Interrupted line shows the result obtained for determining the equilibrium potential of the rectifying current.

shift of the potential it decreased and reversed at about 18 mV. The above result shows that the equilibrium potential for the surge was shifted from -10 mV to $+18$ mV by increasing K concentration from normal to 102·3 mM.

The slowly developing rectifying current appeared when the membrane potential was elevated more than 25 mV and this critical potential level was similar to that observed for the rectifying current in normal sea water. However, the equilibrium potential for the current was elevated by extra K

and coincided with that of the initial surge produced at the same concentration of K. This is shown by a straight line obtained by two-step voltage-clamp technique and denoted by *a* in Fig. 5*C*. Thus the equilibrium potentials for rectifying current and the initial surge also coincide in 102·3 mM-K medium. The present result indicates that this common potential level becomes more positive by increasing K concentration.

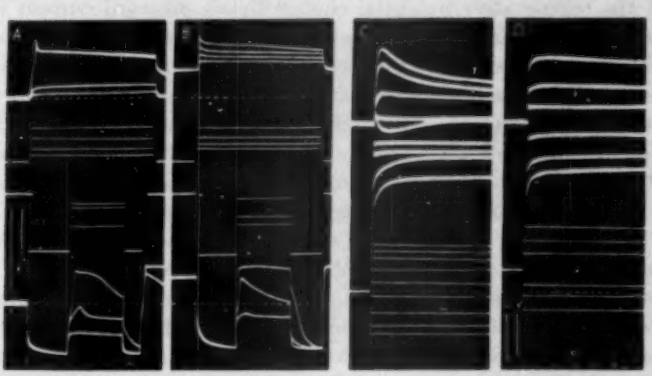

Fig. 6. *A* and *B*, potential changes (uppermost traces) produced by outward current pulses applied to the resting cell membrane (shown in the second group of traces) and those (lowermost traces) by pulses applied to the hyperpolarized membrane (mentioned in the third groups of traces). *A* and *B* were obtained about 1 and 10 min after the start of immersion in KCl sea water (474·3 mM-K and normal [Cl]). The decrease of resting potential from its normal value was about 5 mV in *A* and about 30 mV in *B*. Calibrations: *v*, 50 mV; *c*, 0·2 μA; time marker, 100 msec. *C* and *D*, membrane currents (upper traces) associated with rectangular potential changes (lower traces). The decrease of resting potential was about 5 mV in *A* and about 25 mV in *B*. Calibrations: *v*, 50 mV; *c*, 0·2 μA; time marker, 100 msec.

Figure 6*A* was obtained in KCl sea water while the decrease of the resting potential was about 5 mV. When the depolarization caused by the outward current pulse reached a certain level, the membrane potential suddenly shifted to a new level which was about 40–50 mV more positive than the resting potential. This potential change was usually preceded by a small peak but sometimes no appreciable peak was present. Further increase of the current intensity usually resulted in only a small additional elevation of the membrane potential. In other words, an outward current produced a regenerative potential change which was entirely different from the ordinary spike potential. The effect of the outward current pulse superimposed on a conditioning inward current on the same membrane is given by the lower record in Fig. 6*A*.

As is shown by Fig. 6*C*, the membrane current associated with rect-

angular hyperpolarization or small depolarization was a constant current preceded by a capacitative current. However, a relatively slow inwardly directed deflexion appeared following the capacitative current when the depolarization reached a certain level. With further positive shift of the membrane potential the deflexion became smaller and it changed in direction from inward to outward. This presumably corresponded to the initiation of the regenerative potential change by an outward current in the absence of voltage clamp. The current–voltage relation at the peak of the deflexion is shown in Fig. 7I by crosses and the interrupted line. The relation was linear for hyperpolarization and for small depolarizations, but deviated from the straight line when the membrane potential was shifted from the original normal resting potential by more than 25 mV. The current at the peak of the deflexion became inward with further positive shift of the membrane potential, but again became outward at 45 mV. The result indicates that an increase of the membrane conductance occurred, with an equilibrium potential which was in this case about 45 mV more positive than the resting potential. The present conductance increase is considered to be similar to that associated with the delayed rectification in the normal membrane. Since in the present case the threshold potential for the conductance increase was substantially lower than the equilibrium potential, it resulted in a regenerative potential change instead of a simple rectification.

When the relation was measured 0·6 sec after the start of the voltage change (filled circles and solid line) the deviation from the straight line relation was markedly reduced. This indicates that the above conductance increase decayed with time. The current–voltage relation under a conditioning hyperpolarization was also observed and the equilibrium potential for the surge coincided with that found for the conductance increase associated with a rectangular depolarization (Fig. 7I, open circles). The absolute membrane potential at which the surge appeared was similar to that observed in the normal membrane.

After the resting potential had decreased substantially the regenerative potential change was no longer produced by an outward current (Fig. 6*B* upper) while the break response was still observed (Fig. 6*B* lower). The corresponding current–voltage relations are illustrated in Figs. 6*D* and 7II. They show that only a small conductance increase was associated with a rectangular depolarization, while the initial surge was the same as before. Even though the resting potential was decreased the absolute membrane potential at the threshold for the surge and the equilibrium potential level of the surge were not altered. Therefore, a relatively large conditioning hyperpolarization was necessary to produce a break response in such a depolarized membrane. Figure 8 shows the recordings of the

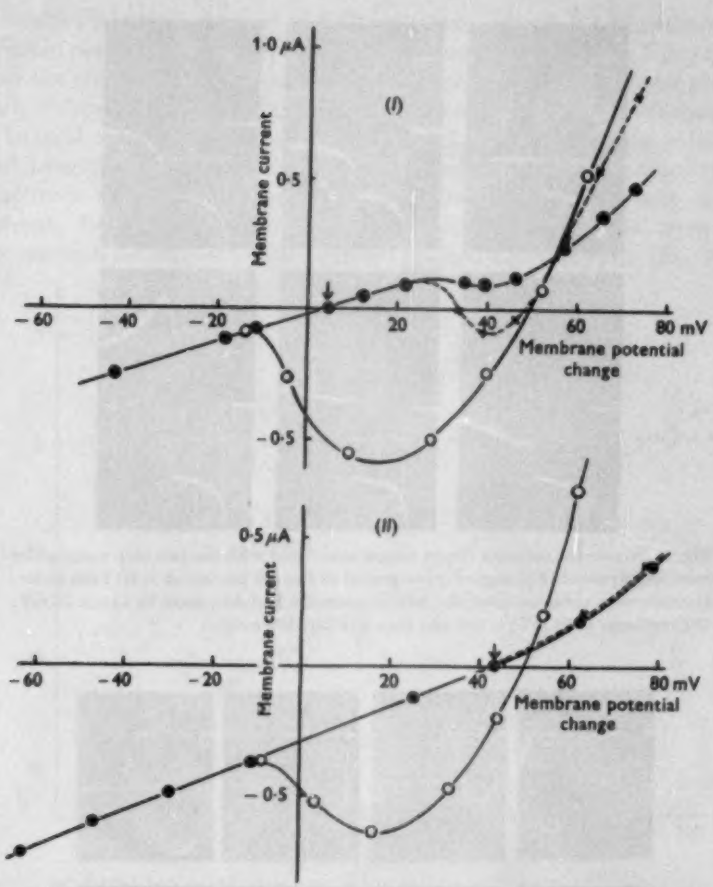

Fig. 7. Current–voltage relations of cell membrane in KCl sea water (474·3 mM-K and normal [Cl]). Membrane potential was measured from the resting potential of the cell immersed in normal sea water. Crosses and filled circles give the relations obtained by a single-step voltage clamp respectively at the peak of the initial slow deflexion, and 0·6 sec after the start of the voltage pulse. Open circles indicate those at the peak of the initial surge when the conditioning hyperpolarizations were 75 mV in (I) and 110 mV in (II). The set of relations (II) was obtained some time after the set of relations (I). The decrease of resting potential had taken place within this period. An arrow in each set indicates the resting potential level at the time of measurement.

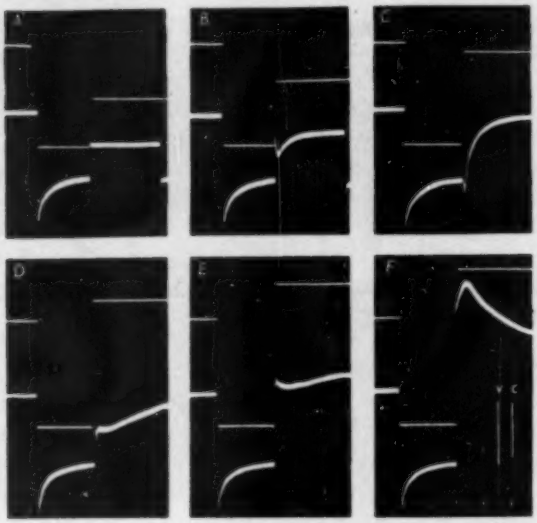

Fig. 8. Membrane currents (lower traces) associated with the two-step rectangular membrane potential changes (upper traces) of the cell immersed in KCl sea water. Records were obtained after the resting potential had decreased by about 25 mV. Calibrations: v, 50 mV; c, 0·2 μA; time marker, 100 msec.

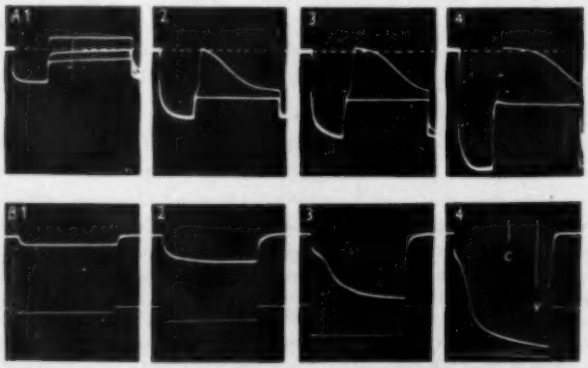

Fig. 9. *A*, effect of the amplitude of conditioning hyperpolarization on the critical potential level for the break response of a cell immersed in KCl sea water. Records were obtained after the decrease of the resting potential had reached a steady state. Calibration, 50 mV; time marker, 100 msec. *B*, potential changes (upper traces) of a cell membrane produced by inward current pulses (shown in lower traces) when hyperpolarizing responses were present. Records were obtained in KCl sea water after the resting potential had fallen to a steady state. Calibrations: v, 50 mV; c, 0·1 μA; time marker, 100 msec.

actual initial surge obtained in KCl sea water. Figure 9*A* illustrates the effect of conditioning hyperpolarizations of varying amplitude. They show that the critical level at which the break response was elicited was practically independent of the amplitude of the conditioning hyperpolarization.

In most cases an inward current pulse applied after the resting potential had been decreased in KCl sea water produced hyperpolarization of an amplitude which was approximately proportional to the intensity of the current. In a few cases, however, 'hyperpolarizing responses' were observed when the intensity of the current reached a certain value (Fig. 9*B*);

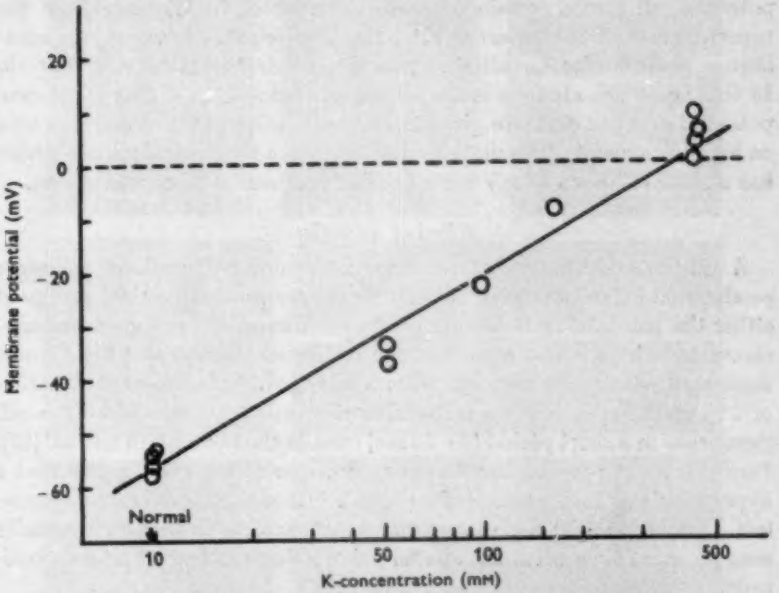

Fig. 10. Relations between the equilibrium potential of the initial surge (ordinate) and the K concentration in the applied solution (abscissa). The former was measured from the reference potential level; the latter is on a logarithmic scale.

the rate of fall of the potential changed and the amplitude of the hyperpolarization became much larger than that expected from the current–voltage relation for a small current intensity. When the membrane resistance was examined by superposing small current pulses on the inward current it was always found that the response was associated with a decrease of membrane conductance. Similar responses have been observed in the squid axon (Segal, 1958; Moore, 1959; Tasaki, 1959) and in the nodal membrane of a myelinated fibre (Stämpfli, 1958; Tasaki, 1959) when immersed in K-rich solutions. In the *Onchidium* nerve cell, however, a clear

hyperpolarizing response was observed for only a short period after a relatively fast decrease of the resting potential. In such cases an increase of the membrane conductance caused by the depolarization probably remained and the hyperpolarizing response is considered to represent an abolition of this conductance increase.

The foregoing results show that the four characteristic levels of the membrane potential, the equilibrium potential for the slowly developing rectifying current, that for the initial surge with a conditioning hyperpolarization, the peak level of the break response and that of the after-potential, all give a certain common value which is determined by the concentration of the external K^+ ions. The relation between the equilibrium potential for the initial surge and concentration is shown in Fig. 10. In this figure the abscissa indicates the absolute value of this membrane potential and the ordinate gives the concentration of the potassium ions on logarithmic scale. The plots approximate to a single straight line which has a slope of about 35 mV for a ten-fold increase of K concentration.

Effect of narcotics

A cell immersed in normal sea water containing 2% urethane no longer produced a spike potential, though there was no appreciable change of either the potential or resistance of the membrane. By using the voltage-clamp technique it was seen that the rectifying current and the current associated with break response were unchanged. Subsequent application of 2% urethane in KCl sea water almost completely depolarized the cell membrane in a short period (40–50 sec) even in the presence of normal [Cl]. During a short period after the quick decrease of the resting potential a hyperpolarizing response was often found. Although it usually soon became less marked, a slight decrease of the membrane conductance was usually seen for some time when the anodal polarization reached a certain amplitude.

When an outward current pulse was applied to the urethane-KCl-treated cell membrane, hyperpolarized by an inward current of a sufficient intensity, a prolonged break response appeared (Fig. 11, *A*, 1). When the urethane-KCl sea water was replaced with normal KCl sea water a few characteristic changes were observed. The resting potential usually increased slightly, the resting membrane conductance increased and the duration of the break response produced by a short outward current pulse became shorter (Fig. 11, *A*, 2). The current–voltage relations in the urethane-KCl and KCl sea water are given in Fig. 12. The abscissa in these figures indicates the membrane potential measured from the resting potential level in the normal sea water. The relation at the steady state is given by filled circles and at the peak of the initial surge by open circles. The

results show that the current at the steady state as well as at the peak of the surge is decreased in the presence of urethane. However, the duration of the initial surge was much prolonged in urethane-KCl solution. The current–voltage relation of the membrane was still quite far from the steady state in the urethane solution after 250 msec, while it had almost reached the steady-state level in KCl sea water (crosses in Fig. 12, I and II). This presumably corresponded to the prolonged break response of the

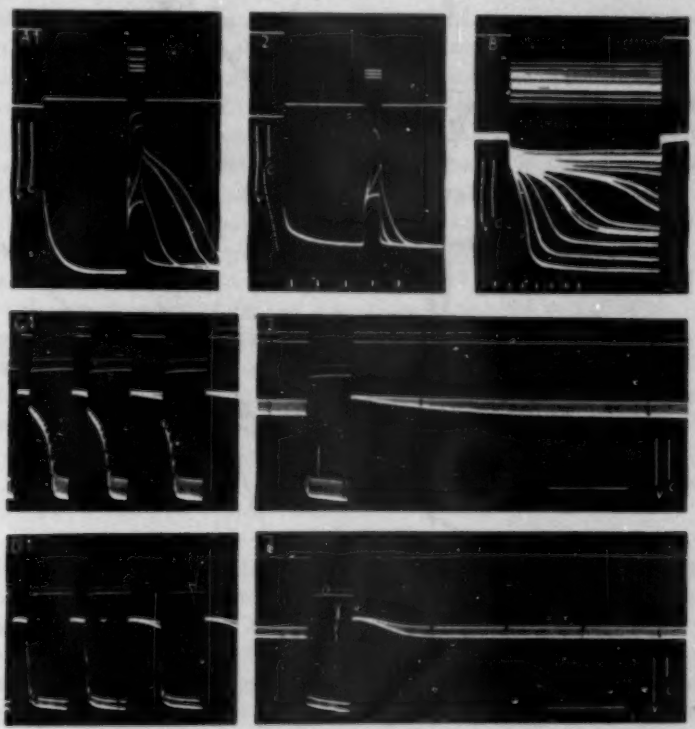

Fig. 11. *A*, break responses (shown in lower traces) produced by short current pulses (shown in upper traces) in a KCl sea water containing 2% urethane (*A* 1), and after the cell had been brought back to normal KCl sea water (*A* 2). Calibrations: *v*, 50 mV; *c*, 0·2 μA; time marker, 100 msec. *B*, membrane potential changes produced by inward current pulses of various intensities applied to a cell immersed in a KCl-sea water containing 2% urethane. Each pulse was applied while the membrane conductance was increased by a preceding large inward current pulse. Calibration: *v*, 50 mV; *c*, 0·2 μA; time marker, 100 msec. *D* and *E*, conductance changes during and after the application of inward current pulses. A series of short outward current pulses were superposed on the inward currents (shown in upper traces). Sets of record *C* and *D* were obtained in KCl sea waters with and without 2% urethane respectively. Calibration: *v*, 50 mV; *c*, 0·2 μA; time marker, 1 sec.

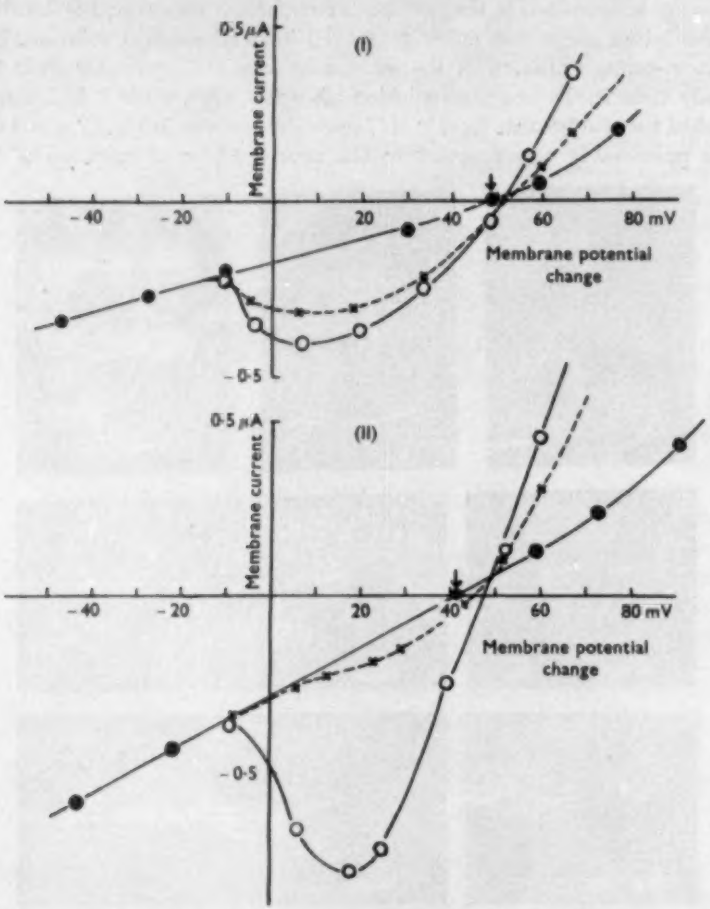

Fig. 12. Current–voltage relations of a cell membrane in a KCl sea water containing 2% urethane (I) and after washing the preparation with normal KCl sea water (II). Membrane potential was measured from the resting potential of the cell when immersed in normal sea water. The resting potentials in the test solutions were indicated by arrows. Filled circles give the relation at the steady state of a single-step voltage clamp. Open circles and crosses show those obtained by two-step voltage clamp at the peak and 250 msec after the peak of the initial surge respectively. The conditioning hyperpolarization was 95 mV in (I) and 85 mV in (II).

urethane-treated cell in the absence of a voltage clamp. The steady-state current–voltage relation was linear for the hyperpolarization in the KCl sea water but a slight non-linearity was usually found when urethane was present. The slope of the relation was slightly larger for a smaller hyperpolarization and this corresponded to the fact that the resting membrane conductance was slightly higher than that of the hyperpolarized membrane.

To measure the membrane conductance a series of small outward current pulses were applied to the membrane during and after the anodal polarization. Figure 11*C* and *D* show the results obtained in urethane-KCl and KCl sea waters respectively. In both cases an increase of conductance was observed at the break of the anodal current but the change lasted much longer in urethane-KCl sea water. These records also show that the anodal polarization decreased the membrane conductance in the urethane solution. Even when an anodal current was applied during the period of increased membrane conductance a similar decrease was seen. A certain threshold value of hyperpolarization was found for this conductance decrease and this is shown in Fig. 11*B*. This record was obtained by applying inward current pulses of various intensities during the period of increased membrane conductance. The phenomenon seems to be similar to the hyperpolarizing response described before.

Cocaine (0·5%) also diminished the spike potential of the cell when added to the sea water. However, the effect on the break response was a simple depression in the normal as well as K-rich media.

DISCUSSION

In the previous paper (Hagiwara & Saito, 1959*b*) it was shown that the membrane current associated with a rectangular change of the cell membrane potential in normal sea water is characterized by two component currents; one is related to the rise of spike potential and the other to the slowly developing rectifying action of the cell membrane. The former is associated with a conductance increase of the membrane to an equilibrium potential which is similar to the peak level of the spike potential, and the latter is associated with a conductance increase to an equilibrium potential which corresponds to the potential level at the peak of the positive after-potential. These two currents correspond to those referred as the sodium and potassium currents in the sodium theory (Hodgkin & Huxley, 1952*a*, *b*).

Beside these conductance changes the present results show that a third conductance increase is present during a conditioning hyperpolarization and that this is related to the 'break response' of the cell. The equilibrium potential of the third conductance increase is similar to that of the

rectifying current. Although the equilibrium potentials are the same, the third conductance increase is usually very transient while the rectification lasts much longer. However, the slowly developing rectifying current also shows a certain slow decay when the membrane potential is maintained at high level for a long time. This becomes more marked when observed in K-rich media.

The common equilibrium potentials for these conductance changes are determined by the concentration of the potassium ions in the external solution. On the other hand, the resting potential depends on the chloride concentration as well as on that of potassium. After the resting potential decreased in the potassium-rich media the break response was observed, but no conductance change was usually associated with depolarization. This indicates that the conductance increase associated with the slowly developing rectifying action has already decayed during the maintained low resting potential. The hyperpolarizing response sometimes observed probably occurs before the decay of this conductance increase and therefore it represents a reversed reaction of the break response.

These results indicate that the cell membrane undergoes a change from one state to the other by the shift of the membrane potential. In the one state the cell membrane shows an increased conductance to an equilibrium potential which is determined by the K concentration. Therefore the cell membrane is more dependent on the K concentration at this state. In the other state the membrane is less dependent on K concentration. By analysing similar threshold phenomena in potassium-rich media Stämpfli (1958) and Moore (1959) attributed these two membrane states to changes in the specific membrane permeabilities to K and Cl ions respectively. On the other hand, Tasaki (1959) has explained these states in K-rich media as well as the threshold phenomena in the normal media with a theory of two stable states.

The conductance change associated with the break response has not yet been observed in other excitable tissues. This conductance change and that associated with the delayed rectifying action may take place in different parts of the cell membrane. However, at present, we have too little evidence to reach a definite conclusion on this point.

SUMMARY

1. The current–voltage relations of the nerve cell membrane of *Onchidium verruculatum* were investigated when the potassium concentration of the external medium was altered.

2. An outward current pulse applied to the hyperpolarized cell membrane produced a 'break response' when the membrane potential reached

a certain critical level. The conductance increase associated with the break response had an equilibrium potential which was similar to that of the increase associated with the delayed rectification of the membrane and also to the peak of the after-potential of the spike.

3. This common potential level was determined exclusively by the K concentration of the external medium, while the resting potential depended on both the Cl and K concentrations.

4. When the resting potential was decreased in K-rich media an inward current of sufficient intensity sometimes produced a 'hyperpolarizing response', which represented a reversed reaction of the break response.

5. Urethane did not depress but prolonged the break response, while the effect of cocaine was a simple depression of the response.

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RELATION BETWEEN THE COLLIGATIVE PROPERTIES AND CHEMICAL COMPOSITION OF SWEAT

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Of all the solutes so far identified in sweat quantitatively the most important are sodium, potassium, chloride, lactate, urea, and ammonia. Sodium and chloride are nearly always the most abundant of these solutes. Although workers at the beginning of the century (Ardin-Deltiel, 1900; Bogdan, 1904; Strauss, 1904) attempted to determine a relationship between the sodium chloride concentration of sweat and its osmotic or more strictly its colligative properties, it was not until 1949 that a comparison was made by van Heyningen (1949) between the concentrations of all these constituents taken together and the osmotic pressure of sweat. More recently Lichton (1957), and Adams, Johnson & Sargent (1958) again investigated this relationship. Whilst van Heyningen and Lichton concluded from their analyses and calculations that they were able to account for the major portion of the colligative properties of sweat in terms of known constituents, Adams *et al.* were unable to confirm these results. In fact under certain conditions they concluded that a very considerable proportion of the osmotic pressure of sweat remained unaccounted for. In view of this disagreement the relationship has been examined in this investigation.

THEORETICAL CONSIDERATIONS

In order to understand the nature of the problem involved, and to correct misconceptions which seem to exist amongst some workers in this field, a brief discussion of aspects of the theory relating to the colligative properties of solutions is presented.

From purely thermodynamic considerations it can be shown that the following relations apply to very dilute and ideal solutions:

$$\pi = RTm;$$
$$\Delta T = Km;$$

where π = osmotic pressure,
R = gas constant,
T = absolute temperature,
m = molality,
ΔT = depression of freezing point of solvent in solution, and
K = cryoscopic constant.

Whilst sweat solutions are very dilute, they are not ideal in behaviour. Although an ideal solution, like an ideal gas, is purely a concept, with dilute non-electrolyte solutions deviations from ideality are so small that the behaviour of these solutions can be considered as ideal. With electrolyte solutions, however, deviations from ideality are considerable, even with solutions as dilute as sweat.

In order to deal with the properties of non-ideal solutions in terms of thermodynamically derived relationships the important concepts of the activity and osmotic coefficients have been introduced. Both these coefficients represent departure from ideal behaviour and one of them must always be introduced with concentration terms when applying exact thermodynamic relationships to solutions which show marked deviations from ideality. As both these coefficients are correction factors they must be assessed by direct determination and their values so obtained only apply to the conditions under which they were determined.

The osmotic coefficient is so defined that the corrected osmotic-pressure and freezing-point equations now become:

$$\pi = RT\phi m;$$
$$\Delta T = K\phi m;$$

where ϕ = molal osmotic coefficient.

Again, from purely energetic considerations the osmotic pressure of dilute solutions can be shown to be directly proportional to the depression of the freezing point. Several assumptions are involved in deriving this proportionality, but for nearly all practical purposes the discrepancies due to omitting to take into account these assumptions can be neglected. Further, as no concentration term is involved in this proportionality the relationship applies to non-ideal as well as to ideal solutions.

The most frequently used units of osmotic pressure are osmoles. An osmole is the osmotic pressure which would be exerted by 1 g-molecule of an ideal non-electrolyte in 1000 g of solvent at 0° C (Dick, 1959). Therefore, for an ideal solution π (osmoles) = m, but for a non-ideal solution π (osmoles) = ϕm. An osmole is equal to 22·4 atmospheres, and an osmolar solution with water as the solvent would give a depression of the freezing point of 1·86° C. Thus the constant relating the osmotic pressure (in osmoles) to the depression of the freezing point (° C) becomes 1/1·86 for water as solvent. Because of the non-ideality of solutions, an osmole is not a measure of concentration: hence the use of such expressions so frequently encountered in sweat physiology literature as 'milli-osmoles/litre' should be avoided.

It is difficult to make direct osmotic pressure measurements. The colligative properties of solutions are more easily investigated by studying the depression of the freezing point. Osmoles are, however, the most convenient units in which to express the magnitude of the colligative properties. Because of the relationship between the depression of the freezing point and the osmotic pressure the colligative properties of a solution can be assessed from the freezing point data and the results expressed in terms of osmoles.

METHODS

Sweat was collected by enclosing the arm in an impermeable envelope and by draining off the sweat at intervals. Sweating was induced either by placing the subject's feet in a hot water-bath (44° C) or by exposure of the subject to 40° C dry bulb, 32° C wet bulb in a hot chamber. Before each series of collections the arm was rinsed with tap water, dried, and thoroughly washed with distilled water. Directly the sweat had been collected it was stored at − 1° C. A certain amount of skin debris was usually obtained with the samples; this was centrifuged off before analysis.

Analyses of the following substances were carried out on each sample of sweat: sodium and potassium by flame photometry, chloride by the method of Sendroy (1937), urea and

ammonia by Conway's microdiffusion technique (1947), lactate by the method of Barker & Summerson (1941). The pH of the sweat was also determined with a pH meter.

A Fiske thermistor osmometer was used to determine the osmotic pressure. Calibration was carried out with sodium chloride solutions of known concentrations, the values of the osmotic coefficients at these concentrations being obtained from *Electrolyte Solutions* by Robinson & Stokes (1955).

As stated above, it is, strictly, invalid to attempt to calculate the osmotic pressure of a solution from the concentrations of its solutes unless the appropriate osmotic coefficients are known, and these have only been determined for simple one-component systems. No attempt has, therefore, been made to calculate osmolalities from concentrations. Instead, after analysing the sweat samples, synthetic-sweat solutions of identical composition were made up from solutions of sodium chloride, potassium chloride, ammonium chloride, sodium lactate, and urea. The osmolalities of these synthetic-sweat solutions were then compared with those of the actual sweat samples.

RESULTS

Sixty-three samples of sweat obtained from fourteen subjects were analysed. Table 1 gives the values obtained for all the analyses. The sweat samples covered a wide range of concentrations and were all within those ranges previously reported for sweat collected in an arm bag. The osmolalities of the sweat samples (Table 2) were likewise very varied; whilst the majority of the samples were markedly hypotonic to plasma, a few samples were hypertonic to normal plasma values. Considerable variations in the sweat rate occurred (Table 2), i.e. from 4 to 64 ml./hr.

Table 2 also gives the osmolalities of the synthetic-sweat solutions, and a plot of osmolalities of synthetic sweat against osmolalities of real sweat is shown in Fig. 1. An analysis of variance of the regression of this plot was carried out and an F test showed that there was a highly significant correlation at the 0·1% level. The linear relationship between R (osmolality of sweat) and S (osmolality of synthetic sweat) is given by

$$\begin{array}{cc} S = 0{\cdot}1485 & +0{\cdot}9192R \\ (\pm 2{\cdot}4084) & (\pm 0{\cdot}0562) \end{array}$$

where confidence limits are expressed at 95% level of probability. This shows that: (i) the gradient of the line is significantly less than unity, (ii) the line passes significantly close to the origin. Therefore the osmolalities of the synthetic-sweat solutions were significantly lower than those of the sweat solutions, and the discrepancy between the osmolalities of the two solutions was proportional to the osmolality of the sweat.

Defining the osmotic deficit as the difference between the osmolalities of the real sweat and synthetic-sweat solutions, expressed as a percentage of the osmolality of the real sweat, i.e.

$$D = \frac{R-S}{R} \times 100, \quad \text{where } D = \text{osmotic deficit},$$

then the osmotic deficit showed no tendency to vary with the osmolality of the sweat. Because of the near proportionality between S and R a mean value of R from all the results was taken in order to determine the osmotic deficit. The value of this deficit thus estimated is 8·0 with a confidence interval of (12·5, 1·0).

The pH of the individual sweat samples (Table 2) varied between 4·6 and 7·1, the mean value being 5·9. The mean value of the pH for all the synthetic-sweat samples was 6·4. No serious discrepancies should, therefore, have occurred on this account.

TABLE 1. Concentrations of the constituents of sweat*

Subject	No. of samples	Na^+ (m-equiv/l.)	K^+ (m-equiv/l.)	NH_4^+ (m-equiv/l.)	Cl^- (m-equiv/l.)	Lactate (m-equiv/l.)	Urea m-mole/l.
Water-bath†							
C	4	92·9 ± 3·6	5·9 ± 1·0	2·9 ± 0·5	87·3 ± 6·4	15·2 ± 2·6	10·6 ± 1·4
CC	5	40·9 ± 8·3	6·1 ± 1·0	3·4 ± 0·9	35·8 ± 8·8	13·6 ± 3·9	6·9 ± 0·8
FF	18	62·4 ± 5·9	8·1 ± 1·3	2·6 ± 0·7	50·9 ± 6·2	18·3 ± 4·2	6·0 ± 0·8
Hot room†							
A	4	108·5 ± 39·7	8·7 ± 4·1	3·4 ± 1·0	89·9 ± 35·0	24·8 ± 5·2	10·2 ± 2·3
B	2	60·0 ± 27·3	10·0 ± 1·6	4·5 ± 1·1	48·5 ± 28·3	30·8 ± 9·1	13·0 ± 2·5
E	3	29·7 ± 9·8	7·2 ± 0·8	7·1 ± 0·2	24·0 ± 8·7	18·8 ± 1·5	14·1 ± 1·8
F	3	55·0 ± 12·0	8·6 ± 1·0	4·9 ± 1·0	47·5 ± 11·8	22·4 ± 3·0	13·0 ± 3·1
K	8	138·6 ± 21·0	10·1 ± 1·4	3·8 ± 0·6	123·3 ± 20·3	22·4 ± 3·1	13·9 ± 2·1
L	3	104·5 ± 20·3	11·4 ± 1·4	3·2 ± 0·9	96·5 ± 20·4	27·1 ± 3·5	9·9 ± 2·2
P	3	53·1 ± 9·5	9·7 ± 0·2	3·5 ± 0·3	45·2 ± 8·3	17·9 ± 0·9	8·2 ± 0·4
R	3	56·1 ± 16·9	8·0 ± 0·8	3·8 ± 0·7	47·1 ± 15·9	18·1 ± 2·9	10·3 ± 1·0
RR	1	65·0 ± 0·0	11·1 ± 0·0	6·6 ± 0·0	49·0 ± 0·0	30·8 ± 0·0	16·5 ± 0·0
S	3	66·1 ± 15·7	9·4 ± 0·6	3·2 ± 0·5	57·8 ± 15·6	22·0 ± 2·2	12·6 ± 1·1
W	3	80·0 ± 21·9	9·9 ± 0·8	4·0 ± 0·3	68·3 ± 21·8	24·9 ± 1·0	7·8 ± 0·2

* Mean ± S.D. † See Methods.

TABLE 2. Rate of secretion, osmotic pressure and pH of 'synthetic' and real sweats*

Subject	Rate of secretion of sweat (ml./min)	Osmotic pressure (m-osmole) Sweat	Osmotic pressure (m-osmole) 'Synthetic' sweat	pH Sweat	pH 'Synthetic' sweat
C	0·41 ± 0·08	212·0 ± 10·1	197·3 ± 7·0	6·1 ± 0·3	6·4 ± 0·1
CC	0·52 ± 0·21	108·2 ± 6·9	97·7 ± 10·0	6·5 ± 0·1	6·2 ± 0·2
FF	0·69 ± 0·19	144·5 ± 12·3	133·7 ± 10·3	6·1 ± 0·7	6·4 ± 0·2
A	0·31 ± 0·16	238·6 ± 82·6	225·3 ± 80·0	5·7 ± 0·1	6·3 ± 0·2
B	0·28 ± 0·04	157·3 ± 40·0	151·8 ± 37·8	5·2 ± 0·1	6·6 ± 0·1
E	0·42 ± 0·27	105·2 ± 22·1	93·0 ± 18·1	4·7 ± 0·1	6·4 ± 0·2
F	0·60 ± 0·33	147·2 ± 16·0	138·3 ± 16·5	5·6 ± 0·2	6·4 ± 0·1
K	0·37 ± 0·18	316·1 ± 45·1	287·4 ± 41·9	6·5 ± 0·3	6·3 ± 0·2
L	0·39 ± 0·10	246·8 ± 36·5	231·7 ± 33·0	5·7 ± 0·2	6·4 ± 0·1
P	0·48 ± 0·15	135·8 ± 20·3	126·5 ± 16·3	6·4 ± 0·2	6·6 ± 0·1
R	0·42 ± 0·24	149·3 ± 33·5	131·8 ± 29·3	5·5 ± 0·1	6·5 ± 0·0
RR	0·06 ± 0·0	191·5 ± 0·0	162·0 ± 0·0	5·2 ± 0·0	6·5 ± 0·0
S	0·32 ± 0·11	174·2 ± 36·5	157·5 ± 25·8	5·3 ± 0·0	6·5 ± 0·1
W	0·26 ± 0·12	195·2 ± 41·5	179·3 ± 43·1	5·0 ± 0·1	6·5 ± 0·1

* Mean ± S.D.

Of the other substances which have been detected in sweat the most important quantitatively are calcium, sulphate, amino acids, and to a less extent reducing sugars, bicarbonate, and creatinine. Although these occur in small amounts compared with the five major constituents, they would on the average account for at least 4 % of the total osmolality (Robinson & Robinson, 1954). If this portion is added, at least 96 % of the osmotic pressure of sweat has been accounted for in terms of known constituents.

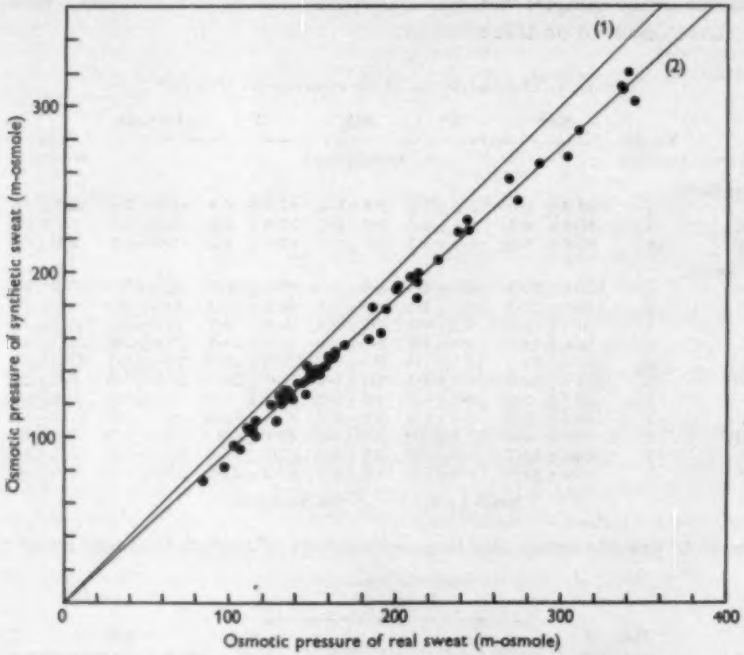

Fig. 1. Relation between osmotic pressure of real sweat and of 'synthetic' sweat solutions. (1) Regression line if no osmotic deficit had occurred. (2) Regression line obtained.

The sums of the measured cations and anions in mg-ions/l. are given in Table 3, and the difference between these two sums (ionic deficit) is expressed as a percentage of the total ionic and molar concentrations (total 'molarity'). The value of this deficit ranged from a cationic deficit of 6·2 % to an anionic deficit of 4·5 %. The mean value of this deficit was, however, an anionic deficit of only 1·2 %.

If osmotic coefficients are neglected and the total 'molarity' (Table 3) is compared with the osmolality for all the solutions, no over-all deficit was obtained.

TABLE 3. Cationic and anionic concentrations, ionic deficit, and total molarity*

Subject	Sum of cations (mg-ions/l.)	Sum of anions (mg-ions/l.)	Ionic deficit (% total 'molarity')	Sum of constituents (m-mole/l.)
C	101·8 ± 3·2	102·5 ± 4·7	−0·3 ± 0·8	214·5 ± 7·9
CC	56·3 ± 16·9	49·3 ± 4·9	+0·8 ± 1·6	106·8 ± 10·8
FF	73·1 ± 5·6	69·2 ± 5·8	+2·6 ± 1·1	148·3 ± 11·2
A	120·6 ± 44·1	114·7 ± 40·2	+2·2 ± 0·8	245·5 ± 86·6
B	74·5 ± 24·6	79·3 ± 19·2	−3·4 ± 4·0	167·0 ± 41·0
E	44·0 ± 10·0	42·8 ± 8·7	+1·6 ± 0·4	101·0 ± 18·7
F	68·5 ± 10·2	69·9 ± 8·9	−1·5 ± 0·7	151·3 ± 16·5
K	152·5 ± 22·3	145·7 ± 20·9	+2·2 ± 0·7	312·0 ± 44·6
L	119·0 ± 18·6	123·6 ± 18·2	−1·9 ± 0·5	252·7 ± 35·1
P	66·3 ± 9·3	63·1 ± 7·9	+2·3 ± 0·8	137·7 ± 17·5
R	67·9 ± 16·6	65·2 ± 13·9	+1·6 ± 1·8	143·3 ± 31·3
RR	82·7 ± 0·0	79·8 ± 0·0	+1·6 ± 0·0	179·0 ± 0·0
S	78·8 ± 16·0	79·8 ± 13·7	−0·7 ± 1·5	171·3 ± 29·0
W	93·9 ± 22·9	93·2 ± 22·4	+0·3 ± 0·2	194·7 ± 45·5

* Mean ± S.D.

DISCUSSION

Lichton (1957) collected forty-five samples of forearm sweat in an impermeable envelope from one subject, and analysed them for sodium, chloride, potassium, lactate, ammonia, and urea. Comparing the values of the total 'molarities' and osmolalities obtained for his solutions, and assuming an equivalence between 'molarities' and osmolalities (i.e. neglecting the osmotic coefficients), he claimed to have accounted for all the osmotic pressure of sweat in terms of these constituents. This claim can be supported by results obtained in this investigation.

Using fifty arm-bag samples from each of three subjects van Heyningen (1949) measured the osmotic pressure and determined its chloride, lactate and urea contents. She then assumed equivalence between the chloride plus lactate on the one hand and sodium, potassium, plus ammonia on the other, and further assumed that there would be about 1/10th the amount of potassium compared to sodium present. These assumptions can be regarded as approximately correct. To her data she then applied the following relationship:

$$\pi = \sum_{n} \frac{Kx}{1{\cdot}86}$$

where π = osmotic pressure in osmoles,
K = cryoscopic constant of component,
x = molar concentration of component, and
n = number of measured components,

and thus obtained a mean osmotic deficit of 8%. By applying the same relationship to the present data a mean osmotic deficit of about 4% is

obtained. This relationship does not, of course, allow for the variability of the osmotic coefficient with different concentrations and so is not strictly valid, although it appears to yield a closer approximation than that obtained by totally neglecting the osmotic coefficients and assuming total 'molarity' to be equal to osmolality.

Adams *et al.* (1958) collected 375 samples of arm sweat from 95 men using impermeable gloves and analysed them for sodium, potassium, chloride, lactate and urea. Simply by comparing total 'molarities' with osmolarities (cf. Lichton, 1957) they concluded that 'in about 15% of all samples as much as 25% of the total osmolarity remained to be accounted for'. Sweat samples were collected daily for six weeks, and some of their subjects were maintained during the 3rd and 4th weeks on different dietary regimens. It was from these subjects that they appear to have obtained the most pronounced osmotic deficits, i.e. an average deficit of 12%. For all other subjects average deficits were 7%. The osmolalities of the synthetic-sweat solutions (Table 2) were on the average about 9% less than the total 'molarities'. Using this value to allow for the non-ideality of the sweat collected by Adams *et al.*, their mean osmotic deficits would be of the order of 15% for 'control' subjects and 20% for 'experimental' subjects. Not only does this markedly reduce the apparent differences between their 'experimental' and 'control' subjects, but it shows that there is a marked difference also between their 'control' values and the values obtained in this investigation.

Adams *et al.* found that an over-all anionic deficit occurred for both 'experimental' and 'control' subjects, and that this, as well as the osmotic deficit, was greater for 'experimental' than for 'control' subjects. On the basis of this evidence they postulate the presence in sweat of a hitherto unrecognized 'organic acid of high pK' on the assumption that, at the mean value of the pH of their sweat samples (pH 5), the acid would only be partially ionized and hence its osmotic effect would be greater than its ionic effect. The ionic differences obtained in this investigation (Table 3), however, can readily be explained by the presence of small quantities of known but unanalysed constituents.

What is even more striking about the results of Adams *et al.* is the very marked variation in the value of their osmotic deficit. They state that there were relationships between the rate of secretion of sweat, the osmotic pressure of the sweat, and the osmotic deficit. At high osmotic pressures and low rates of secretion there was a tendency for the osmotic deficit to increase, so that at very high osmotic pressures and very low rates of secretion 40–50% of the total osmolarity was unaccounted for. Their claim was not confirmed by the investigation reported here. Although there were considerable variations in the rate of sweating (Table 2) and

also in the osmolality of the sweat there was (i) no general tendency for the osmotic deficit to increase with low rates of flow, and (ii) no tendency for the osmotic deficit to vary at all with changes in osmotic pressure (Fig. 1).

The results of this investigation confirm those of van Heyningen (1949) and Lichton (1957) in that the major portion of the osmotic pressure of sweat can be accounted for by the presence of known constituents.

SUMMARY

1. Sixty-three samples of sweat from fourteen subjects were analysed: osmolalities of the sweat solutions and of solutions made up containing the analysed concentrations were compared. The mean osmotic deficit thus obtained in terms of the measured constituents was 8%.

2. The results show that together with the other known constituents in sweat the major portion of the colligative properties of sweat can be accounted for in terms of known constituents.

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THE EFFECT OF SOME COFACTORS ON THE SYNTHESIS OF FATTY ACIDS AND CHOLESTEROL IN CELL-FREE PREPARATIONS OF RAT LIVER

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Brady (1958) and Wakil (1958) have shown that cell-free preparations of pigeon liver synthesize long-chain fatty acids through a pathway which includes the formation of malonyl co-enzyme A (CoA) from acetyl CoA and CO_2. It has also been shown that biotin and Mn^{2+} are required for this carboxylation (Wakil, Titchener & Gibson, 1958; Brady, 1958; Wakil, 1958). We have recently shown that the synthesis of cholesterol and fatty acids by cell-free fractions of rat liver can be modified by injecting the rats with thyroxine before the livers are removed (Fletcher & Myant, 1960). When the rats are given 20 μg of thyroxine, there is an increase in cholesterol synthesis and a fall in fatty acid synthesis. With doses of 30 μg or more both syntheses are usually depressed. In order to interpret these results it was necessary to know whether our cell-free fractions of rat liver synthesize fatty acids by the same pathway as that followed in the preparations of pigeon liver used by Brady (1958) and by Wakil (1958). The effect of small doses of thyroxine also raised the question as to how far the two biosynthetic pathways are linked by competition for common cofactors or precursors. If they are so linked, it might be expected that when the rate of one biosynthesis is modified, that of the other would tend to change in the opposite direction. In this paper we describe the effect of various cofactors on the synthesis of fatty acids and cholesterol in cell-free fractions of rat liver.

METHODS

The following abbreviations are used in this paper: DPN and DPNH, diphosphopyridine nucleotide in oxidized and reduced form; TPN and TPNH, triphosphopyridine nucleotide in oxidized and reduced form; ATP, adenosine triphosphate; G6P, glucose 6-phosphate.

The rats were albino males of an inbred strain weighing 120–150 g. They were killed by a blow on the head and the livers were removed at once and homogenized by the technique of Bucher & McGarrahan (1956), modified by Popják, Gosselin, Gore & Gould (1958), in ice-cold buffer consisting of 0·1 M potassium phosphate buffer at pH 7·4; 0·005 M-$MgCl_2$; 0·03 M nicotinamide; and 0·003 M glutathione. Nuclei and cell debris were removed from the

homogenate by centrifugation at 2000*g* for 25 min. The mitochondria were then removed from the supernatant by centrifugation at 10,000*g* for 20 min. The resulting supernatant, containing the lighter subcellular particles and the soluble enzymes of the cell sap, is designated S_{10}. In some experiments the lighter particles (microsomes and granules of glycogen) were separated as a pellet by a further centrifugation at 104,000*g* for 60 min. The supernatant (S_{104}) from this spinning was decanted from the pellet (*M*). The pellet was washed by suspending it in a volume of buffer equal to one-fourth the volume of the S_{104} and spinning it at 104,000*g* for a further 60 min. The washed pellet was then suspended in a volume of buffer equal to one-tenth the original volume of S_{104}. S_{104} was also centrifuged a second time to remove any microsomes that had remained in suspension after the first spinning at 104,000*g*. All these operations were carried out at 0° C.

Incubation of tissues. All incubations were carried out for 2 hr at 37° C, with air as the gas phase, in a standard medium consisting of the buffer used for preparing the fractions, with the addition of DPNH (1·0 μmole), TPN (0·5 μmole) and glutathione (20 μmole); other cofactors were added as described under Results. The final volume, including homogenate and buffer, was 3·5 ml. Each flask contained the S_{10} derived from 0·7 *g* of fresh liver. At the end of the incubation, 2 mg of cholesterol was added as carrier to each flask and the contents were hydrolysed by allowing the flasks to stand for 2 hr on a steam-bath after the addition of 1 ml. saturated NaOH and 3 ml. ethanol. In some experiments 40 mg of soap prepared from ox fat was also added as carrier before the hydrolysis. After hydrolysis cholesterol (as digitonide) and fatty acids (as soaps) were isolated and assayed for radioactivity by the methods described elsewhere (Fletcher & Myant, 1960). In some experiments the soaps were redissolved in water and acidified. The free fatty acids were then extracted with petroleum ether and methylated with diazomethane. The methyl esters were separated and their radioactivity determined by gas–liquid radiochromatography, as described by Popják, Lowe, Moore, Brown & Smith (1959). Avidin was prepared from fresh eggs by the method of Woolley & Longsworth (1942).

RESULTS

Effect of cofactors on synthesis of fatty acids and cholesterol from acetate

The effect of the cumulative addition of CoA, ATP, HCO_3^-, malonate and Mn^{2+} on fatty acid and cholesterol synthesis was studied in S_{10} fractions. Table 1 shows the results from a representative experiment. When all five factors were present, synthesis of fatty acids was increased to more than 28 times that observed in the standard medium alone. The effect on cholesterol synthesis bore no consistent relationship to the change in fatty acid synthesis. Cholesterol synthesis was stimulated by CoA, but inhibited by ATP and Mn^{2+}. A similar degree of inhibition of cholesterol synthesis was also observed when Mn^{2+} was added in the absence of CoA, ATP, HCO_3^-, and malonate. The effects of HCO_3^- and malonate were variable. In some cases there was a rise in cholesterol synthesis; in others there was a fall.

Effect of biotin and avidin

Biotin had very little effect on fatty acid synthesis, either in the standard medium alone or in the presence of all the cofactors shown in Table 1. However, it was possible to demonstrate a requirement for biotin by observing the effect of avidin, a specific inhibitor of biotin.

When avidin was added to the incubation medium, fatty acid synthesis was depressed to less than 10% of that observed in the absence of avidin, and the depression was reversed almost completely by the addition of biotin. Table 2 shows an experiment in which the effect of avidin was observed in the presence of the standard medium to which other cofactors were added. In spite of the marked effect on fatty acid synthesis, avidin had no consistent effect on cholesterol synthesis. In the experiment shown in Table 2 there was a slight depression, but in others there was a slight increase, as in Table 3.

TABLE 1. Effect on fatty acid and cholesterol synthesis from acetate of adding cofactors to S_{10} fractions from normal rat liver incubated in standard medium with [1-^{14}C] acetate (25 μc + 20 μmole); CoA, 0·1 μmole; ATP, 10·5 μmole; $KHCO_3$, 35 μmole; K malonate, 25 μmole; Mn^{2+}, 12 μmole. The final volume was 3·5 ml. Values show average μmole ($\times 10^4$) acetate/g wet tissue incorporated into fatty acids and cholesterol in 2 hr in duplicate incubations

Additions to standard medium	Fatty acids	Cholesterol
None	397	269
CoA	934	444
CoA + ATP	976	174
CoA + ATP + HCO_3^-	3023	145
CoA + ATP + HCO_3^- + malonate	8100	151
CoA + ATP + HCO_3^- + malonate + Mn^{2+}	11,130	63

Note. In this experiment ATP was added after CoA, and depressed cholesterol synthesis but had no effect on fatty acid synthesis. When ATP (10·5 μmole) was added without CoA, fatty acid synthesis was always stimulated.

TABLE 2. Effect of avidin on fatty acid and cholesterol synthesis from acetate by S_{10} fractions from normal rat liver. Each flask contained standard medium, with CoA, 0·1 μmole; ATP, 17·5 μmole; G6P, 10 μmole; $KHCO_3$, 35 μmole; K malonate, 25 μmole; [1-^{14}C]acetate (5 μc + 20 μmole). The final volume was 3·5 ml. Avidin and biotin were added in the quantities shown in the Table. Values show average μmole ($\times 10^4$) acetate/g wet tissue incorporated into fatty acids and cholesterol in 2 hr in duplicate incubations

Additions to medium	Fatty acids	Cholesterol
None	6082	505
Biotin (10 μg)	8033	505
Avidin (equivalent to 2 μg biotin)	471	459
Avidin + biotin (10 μg)	5767	471

Effect of HCO_3^-, malonate and Mn^{2+} on cholesterol synthesis in the presence of avidin

The depression of cholesterol synthesis in the S_{10} fraction by ATP and Mn^{2+} (Table 1) might be due either to a direct inhibition of cholesterol synthesis, or to a shortage of common precursors or cofactors resulting from the stimulation of fatty acid synthesis. In an attempt to distinguish between these two possibilities, the effect of these substances was tested in the presence of avidin. In the experiment shown in Table 3 avidin

inhibited fatty acid synthesis, both in the standard medium and in the presence of all the additional cofactors. As in the experiment shown in Table 2, synthesis in the presence of avidin was restored to the control level by the addition of biotin. The inhibitory effect was most marked when synthesis was tested in the presence of all the five factors required for optimal synthesis in the S_{10} fraction. Under these conditions synthesis in the presence of avidin was only 4·3% of that observed in its absence.

TABLE 3. Effect of avidin on the synthesis of cholesterol and fatty acids from acetate by S_{10} fractions from normal rat liver, in the standard medium alone (3·5 ml. final volume, with 5 μc + 20 μmole [1-^{14}C] acetate) and with the addition of cofactors required for optimal synthesis of fatty acids. The cofactors were added in the following amounts: ATP, 10·5 μmole; CoA, 0·1 μmole; $KHCO_3$, 35 μmole; K malonate, 25 μmole; Mn^{2+}, 12 μmole; avidin equivalent to 2 μg biotin; biotin, 10 μg. Values show average μmole ($\times 10^4$) acetate/g wet tissue incorporated into fatty acids and cholesterol in 2 hr in duplicate incubations

Additions to standard medium	No avidin		With avidin	
	Fatty acids	Cholesterol	Fatty acids	Cholesterol
None	584	388	172	462
ATP	1333	193	187	206
ATP + CoA	1331	327	432	339
ATP + CoA + HCO_3^-	3682	337	457	385
ATP + CoA + HCO_3^- + malonate	6185	246	331	317
ATP + CoA + HCO_3^- + malonate + Mn^{2+}	9702	67	419	90
ATP + CoA + HCO_3^- + malonate + Mn^{2+} + biotin	—	—	10,087	68

The effect of the additional cofactors on cholesterol synthesis was independent of the amount of fatty acid synthesized. This is most clearly seen in the case of ATP and Mn^{2+}, each of these cofactors inhibiting cholesterol synthesis to about the same extent in the presence of avidin as in its absence.

Synthesis of fatty acids in S_{104} and M fractions

In cell-free preparations of rat liver, cholesterol synthesis cannot take place in the absence of the microsome fraction (Popják *et al.* 1958). Wakil, Porter & Gibson (1957), on the other hand, found that all the enzymes needed for fatty acid synthesis in cell-free preparations of pigeon liver were in the soluble fraction. We have compared synthesis of fatty acids and cholesterol in the S_{104} of rat liver with that in the combined S_{104} and *M* fractions, in the standard medium and in the presence of the cofactors required for optimal synthesis of fatty acids. The amount of fatty acid isolated from the S_{104} alone was small and rather variable (0·5–2·0 mg). This introduced an error into the comparison, since the amount of fatty acid synthesized is proportional to the product of specific activity and amount isolated. In order to overcome this difficulty, a large excess of soap (40 mg) was added to the flasks at the end of the incubation,

so that the total amount of soap isolated was about the same in all cases. Cholesterol (2 mg) was also added, as in the previous experiments. In both the standard and the optimal medium synthesis of fatty acids always occurred in the S_{104} alone, though the amount was less than half that observed in the combined S_{104} and *M* fractions (Table 4). In three experiments the S_{104} synthesized an average of 38% as much fatty acid as the $S_{104}+M$ in the standard medium, and 24% as much in the optimal medium. Synthesis of fatty acids in the S_{104} was not due to contamination of this fraction by microsomes from the *M* pellet, since the S_{104} did not synthesize any cholesterol (Table 4).

TABLE 4. Synthesis of fatty acids and cholesterol from acetate in S_{104} and *M* fractions from normal rat liver with and without cofactors required for optimal synthesis of fatty acids. Samples were incubated with [1-^{14}C] acetate (15 μc + 20 μmole) either in the standard medium, or in the standard medium with CoA, 0·1 μmole; ATP, 10·5 μmole; G 6P, 10 μmole; $KHCO_3$, 35 μmole; K malonate, 25 μmole; Mn^{2+}, 12 μmole (optimal medium). In each case the final volume was 3·5 ml. Each value shows average μmole ($\times 10^4$) acetate/g wet tissue incorporated into fatty acids and cholesterol in 2 hr in a set of 4 incubations

Medium	Fraction	Fatty acids	Cholesterol
Standard	S_{104}	98	0
	$S_{104}+M$	225	511
Optimal	S_{104}	2143	0
	$S_{104}+M$	8571	159

TABLE 5. Percentage of total radioactivity in fatty acids of different chain length synthesized from [1-^{14}C] acetate in S_{10} fractions of normal rat liver. Fatty acids converted to methyl esters before analysis on adipate column (Popják *et al.* 1959) at 197° C

	Chain length of fatty acid		
Medium*	C_{12}–C_{14}	C_{16}	C_{18}–C_{20}
Standard	62	20	13
Optimal	44	50	6

* Standard medium and optimal medium are as described in Table 4.

Length of carbon chain of fatty acids synthesized

Synthesis of fatty acids by the malonyl CoA pathway in cell-free preparations of pigeon liver results in the formation of long-chain acids (Gibson, Titchener & Wakil, 1958). On the other hand, in the preparations of mammalian tissue studied by Hele, Popják & Lauryssens (1957) and by Seubert, Greull & Lynen (1957), synthesis of fatty acids was considered to take place by the reversal of β-oxidation and resulted in the formation mainly of short-chain acids. It was of some interest, therefore, to test our preparations for synthesis of long-chain fatty acids. Accordingly, the radioactive fatty acids obtained from some of the experiments with S_{10} fractions were analysed by gas-liquid radiochromatography. Although

the greater part of the total activity was in the C_{12}, C_{14} and C_{16} fatty acids, small amounts of activity were also present in the C_{18} and C_{20} acids (Table 5) in the standard medium and in the presence of the cofactors required for optimal synthesis of fatty acids.

DISCUSSION

The stimulating action of bicarbonate and Mn^{2+}, and the inhibitory action of avidin, show that fatty acids are synthesized in the S_{10} fractions of rat liver by a pathway similar to that followed in the soluble extract of pigeon liver studied by Gibson *et al.* (1958). The formation of long-chain radioactive fatty acids in the S_{10} fractions also supports this conclusion, since the yield of C_{16} and C_{18} acids is negligible when fatty acids are synthesized by the reversal of β-oxidation (Seubert *et al.* 1957). Brady (1958) and Gibson *et al.* (1958) found that all the enzymes necessary for the synthesis of fatty acids by the malonyl CoA pathway in pigeon liver were present in the soluble fraction. In our preparations of rat liver, however, synthesis is much increased by the addition of microsomes to the S_{104} fraction. As Ganguly (1960) has pointed out, the intracellular distribution of the enzymes concerned in fatty acid synthesis varies widely from one species to another.

The stimulating action of non-radioactive malonate, first observed by Popják & Tietz (1955) in rat mammary gland, is not easy to understand. Wakil & Ganguly (1959) have suggested that the fatty acid molecule is formed by the successive addition of units of malonyl CoA to a unit of acetyl CoA. Malonate might, therefore, be expected to increase the incorporation of ^{14}C-acetate into fatty acids if the liver contains an enzyme catalysing the conversion of free malonate to malonyl CoA. However, there is no evidence for the presence of a malonate-activating enzyme in rat liver. Moreover, if malonate stimulates fatty acid synthesis by increasing the amount of malonyl CoA, the stimulatory effect should not be inhibited by avidin, since this substance acts only upon the carboxylation of acetyl CoA to malonyl CoA; Table 3 shows that inhibition by avidin is as great in the presence of malonate as in its absence. One way of explaining the action of malonate is to suppose that rat liver contains an enzyme which breaks down malonyl CoA and that this is inhibited by free malonate. There is, however, no evidence for this.

The inhibitory effect of ATP and Mn^{2+} on cholesterol synthesis does not seem to be due indirectly to stimulation of fatty acid synthesis, since the effect still occurs when fatty acid synthesis is almost abolished by avidin, though avidin itself does not inhibit cholesterol synthesis. It is likely, therefore, that in the concentrations tested here these substances have a direct inhibitory action on cholesterol synthesis. The inhibition of

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The inhibitory effect of ATP and Mn^{2+} on cholesterol synthesis does not seem to be due indirectly to stimulation of fatty acid synthesis, since the effect still occurs when fatty acid synthesis is almost abolished by avidin, though avidin itself does not inhibit cholesterol synthesis. It is likely, therefore, that in the concentrations tested here these substances have a direct inhibitory action on cholesterol synthesis. The inhibition of

cholesterol synthesis by ATP is in agreement with the results of Knauss, Porter & Wasson (1959), who found that cholesterol synthesis reached a maximum when the concentration of added ATP was about 1 μmole/ml. and that the amount synthesized was much reduced when the ATP concentration was increased to 3 μmole/ml., the concentration used here.

The observation that a marked reduction in fatty acid synthesis makes little difference to the amount of cholesterol synthesized (Tables 2 and 3) suggests that in the conditions of these experiments cholesterol synthesis is largely independent of fatty acid synthesis. However, it cannot be assumed that in the intact cell, under physiological conditions, the pathways for fatty acid and cholesterol synthesis are as independent of one another as they appear to be in the S_{10} fractions we have studied. In our experiments acetate was added in excess, and this may also be true of some of the cofactors common to the two biosyntheses. In conditions in which the concentration of one or other of these was rate-limiting, competition might be expected to occur.

SUMMARY

1. The effects of certain cofactors upon the synthesis of fatty acids and cholesterol in a cell-free preparation of rat liver are described.

2. Fatty acid synthesis from acetate was stimulated by adenosine triphosphate, HCO_3^-, malonate and Mn^{2+}, and was inhibited by avidin. This suggests that fatty acids were synthesized in this preparation via the 'malonyl-CoA' pathway, known to occur in pigeon liver and other tissues.

3. When fatty acid synthesis was stimulated by ATP and Mn^{2+}, cholesterol synthesis was depressed. This effect is not due to competition between the two biosyntheses for common precursors or cofactors, since the same depression was observed when fatty acid synthesis was inhibited by avidin. It is concluded that, in the concentrations tested, ATP and Mn^{2+} directly inhibit cholesterol synthesis.

4. Avidin, in concentrations sufficient to inhibit fatty acid synthesis, had little effect on cholesterol synthesis. It is concluded that the amount of cholesterol synthesized is largely independent of fatty acid synthesis in the conditions of our experiments.

We wish to thank Dr G. Popják for interpreting the radiochromatograms obtained from the analysis of fatty acids. We are also indebted to Drs J. Christophe and R. Dils for help.

Note added in proof. Since this paper was submitted for publication, our attention has been drawn to a paper by Bloomfield & Bloch ('The formation of Δ^9-unsaturated fatty acids', *J. biol. Chem.* (1960). **235**, 337–345) describing observations on the synthesis from acetate of cholesterol and fatty acids by whole yeast cells of a biotin-requiring strain. Synthesis of cholesterol was higher in the absence of biotin than

it was when fatty acid synthesis was restored by the addition of biotin. This suggests that in intact yeast cells there may be competition between the two biosynthetic pathways for common precursors or cofactors. As is pointed out in our Discussion, this does not necessarily conflict with our observations on the effect of avidin in the S_{10} fraction of rat liver.

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ROLE OF THE VESTIBULAR APPARATUS IN THE PERCEPTION OF MOTION ON A PARALLEL SWING

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The sensitivity of the normal human being to linear motion has received little study; the only determinations of threshold known to the author are given in Table 1. The origin of the sensations has been a matter of opinion. Neither Delage (1888) nor Travis & Dodge (1928) were convinced that vestibular receptors were important, whilst Bourdon (1914) suggested

Table 1. Thresholds of normal human subjects reported by other investigators

Author(s)	Year	Apparatus	Plane of movement	Acceleration at threshold (cm/sec²)
Mach	1875	Balance	Vertical	12
Delage	1888	Parallel swing	Horizontal	23
Bourdon	1914	Trolley pulled by falling weight	Horizontal	4–15
Travis & Dodge	1928	Oscillating platform	Horizontal	3–16
Kunze quoted by Armstrong	1939	No details	Horizontal	2–20
Jongkees & Groen	1946	Parallel swing	Horizontal	6–15
Graybiel & Patterson*	1955	Human centrifuge	Horizontal	26

* Threshold for the 'oculogravic illusion', the apparent tilt of a source of light observed during rotation. The value has been recalculated from their data to facilitate comparison with the other results.

that the problem should be approached by studying the sensitivity of deaf-mutes. An effort has now been made to settle the question experimentally. To provide the motion use has been made of a parallel swing, a stretcher suspended at its four corners by wires, and an attempt has been made to determine the role of the vestibular apparatus in the sensations set up when the swing oscillates. The influences of vestibular lesions have been investigated as have the effects of total immersion in water and of high spinal lesions. The work may be regarded as an extension of that of Jongkees & Groen (1946), who also used a parallel swing but were interested predominantly in the sensations of tilt that may be aroused with somewhat larger swings than are needed for the sensation of motion.

METHODS

In observations on patients with spinal and vestibular lesions and on normal subjects a parallel swing with wires 1·6 m long was used, giving a period to the motion of 2·5 sec (Fig. 1). Both longitudinal and transverse movements were used; with care yawing motion could be avoided. The person being tested indicated the direction in which he felt himself moving. The oscillations were gradually reduced in amplitude; the least value at which they could be detected was recorded. The mean of four such determinations was taken as threshold. The values are expressed in terms of acceleration and refer to the point at which the swing is changing direction and is momentarily still. Preliminary experiments on the effects of total immersion made use of a cylindrical tank mounted on a trolley which was moved backwards and forwards once every 2 sec along rails by a 1 h.p. electric motor. The subject stood in the tank breathing through a tube and indicated by a stick the direction of the motion. The method finally evolved employed the parallel swing method; a galvanized iron bath was supported at its four corners by wires giving a periodicity, again, of one cycle in 2·5 sec (Fig. 2). The subject lay totally immersed breathing through a tube, or tubes with inspiratory and expiratory valves. He was prevented from rising to the surface by padded boards at the head and feet; the direction in which he felt the motion to occur was signalled by tapping. The determinations of threshold with the procedure described above were restricted to transverse oscillations, as longitudinal movements set up disturbing wave motion.

RESULTS

Effects of bilateral lesions of the vestibular apparatus

Apparatus was set up at a school for deaf children; the pupils all had some power of speech. The children were subjected to a Bárány test, being rotated once every 2 sec for 20 sec. The presence of nystagmus after the rotation in either direction indicated that some functional capacity remained in the semicircular canals. If no response was obtained with the head in the normal position further tests were performed with the subject looking at the ceiling. If no nystagmus was then obtained after rotation either clockwise or anticlockwise it was concluded that the vertical as well as the horizontal semicircular canals were non-functional. In all the children used for the investigations audiometric examination had revealed the existence of severe bilateral nerve deafness. In this way four children were selected who were deemed suitable for the investigation. Their ages ranged from 8 to 16. Three of these had suffered from tuberculous meningitis, the fourth from meningococcal meningitis.

The results on the parallel swing were clear-cut; the deaf children with no responses to the Bárány tests had high thresholds; comparison with seven normal subjects aged from 6 to 33 is striking (Table 2). The true values for the sensitivity of the normals may be lower even than the figures recorded, for reliable oscillations could not be obtained at sufficiently small amplitudes in all instances. The total swing needed at threshold was often substantially less than 1 cm.

Hereditary deafness affords an opportunity for separating the effects of

the saccules from the utricles, for in the common variety the pars inferior of the labyrinth consisting of the cochlea and saccule fail to develop normally whilst the utricle and semicircular canals are histologically normal (Fraser, 1932). In five of the children at the school there was a history of hereditary deafness. In each of them the deafness was associated with functional activity of the semicircular canals as shown by the occurrence of nystagmus after rotation. Their thresholds were elevated though less than in the previous group. The view that both saccule and utricle are concerned in the perception of linear motion could account for these findings (Szentágothai, 1952).

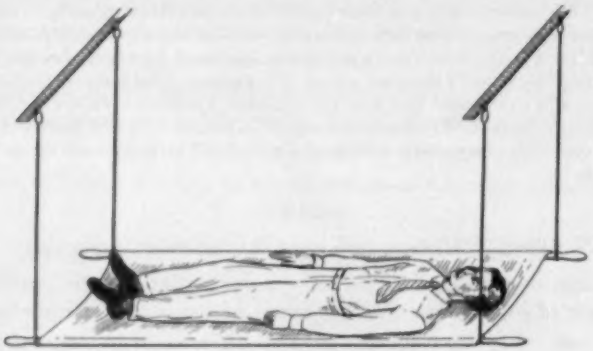

Fig. 1. Subject lying on parallel swing. The rubber or plastic mattress used is not shown. The eyes were closed and covered with a black cloth.

It is arguable that some of the differences are due to a lack of training of the deaf children, for the comparison in Table 2 is with a group of normal subjects who had previously received some practice. The deaf children certainly understood what was required of them and appeared to be trying to the limits of their abilities. Repetition of the tests at a later date was undertaken with more than one child and no clear evidence of increased sensitivity was obtained. The values in both groups of deaf children are furthermore higher than those of the untrained normal subjects tested at Stoke Mandeville (p. 511). In view of these considerations it would be unreasonable to dismiss the striking changes seen in the deaf children as being of no consequence.

Effects of total immersion in water

When the body is accelerated by the application of force to the surface, the extent to which somatic receptors will be stimulated depends on the area over which the force is applied; the possibility that motion is being

detected by somatic rather than by the vestibular receptors must always be considered. A given force is less likely to stimulate somatic receptors when it is spread over as wide an area as possible. Accordingly, immersion in water would be expected substantially to raise the threshold if somatic receptors alone were concerned. In the experiments with the cylindrical tank of water moved on the trolley eight subjects aged 17 to 40 were used. The mean threshold was $2{\cdot}2 \pm 1{\cdot}9$ cm/sec^2 for motion in a sagittal direction and $2{\cdot}4 \pm 1{\cdot}5$ cm/sec^2 for motion transversely. With the rectangular bath eleven subjects were used aged 18 to 37. The mean values for detecting transverse motion was $6{\cdot}4 \pm 2{\cdot}9$ cm/sec^2 in the prone position

TABLE 2. Influence of bilateral vestibular lesions on sensitivity to linear motion

		Thresholds (cm/sec^2)		
	Position	Normals	Complete bilateral loss	Incomplete bilateral loss (hereditary deafness)
Motion in long axis of body	Supine	$2{\cdot}5 \pm 2{\cdot}2$ (7)	$21{\cdot}1 \pm 6{\cdot}1$ (4)	$11{\cdot}2 \pm 5{\cdot}3$ (5)
	Prone	$1{\cdot}9 \pm 1{\cdot}0$ (7)	$22{\cdot}3 \pm 19{\cdot}8$ (4)	$9{\cdot}1 \pm 3{\cdot}9$ (5)
	On right	$1{\cdot}9 \pm 1{\cdot}6$ (5)	$20{\cdot}5 \pm 8{\cdot}4$ (4)	$10{\cdot}3 \pm 2{\cdot}5$ (5)
	On left	$2{\cdot}2 \pm 1{\cdot}9$ (4)	$26{\cdot}0 \pm 21{\cdot}0$ (4)	$12{\cdot}0 \pm 3{\cdot}7$ (5)
Motion in transverse direction	Supine	$1{\cdot}9 \pm 0{\cdot}7$ (7)	$17{\cdot}4 \pm 5{\cdot}0$ (4)	$9{\cdot}9 \pm 4{\cdot}1$ (5)
	Prone	$1{\cdot}9 \pm 1{\cdot}0$ (7)	$15{\cdot}5 \pm 7{\cdot}6$ (4)	$8{\cdot}4 \pm 4{\cdot}0$ (5)
	On right	$1{\cdot}9 \pm 1{\cdot}1$ (5)	$15{\cdot}5 \pm 6{\cdot}4$ (3)	$11{\cdot}2 \pm 3{\cdot}2$ (5)
	On left	$1{\cdot}6 \pm 2{\cdot}1$ (4)	$22{\cdot}0 \pm 16{\cdot}0$ (4)	$8{\cdot}4 \pm 3{\cdot}7$ (5)

Figures in parentheses correspond to number of observations on which the calculations of the mean values and standard deviations are based.

and $6{\cdot}1 \pm 2{\cdot}1$ cm/sec^2 supine. Lying on the right, values of $5{\cdot}4 \pm 1{\cdot}8$ cm/sec^2 were obtained as against $5{\cdot}5 \pm 2{\cdot}3$ cm/sec^2 when lying on the left. These values are higher than the control values of Table 2, but the situation is one scarcely favouring a high level of performance in a sensory test. The thresholds compare favourably with those reported by other workers (Table 1) and are about the same as the set of data from the second group of normal observers (p. 511).

Conditions would be ideal if the body were of uniform density and if it were immersed far below the surface in a liquid of appropriate specific gravity so as to be accurately 'trimmed'. These conditions cannot be fulfilled. As the density of the chest changes with respiration no amount of trimming is likely to yield more satisfactory conditions; some downward force must be exerted. At the regions of contact of the head and feet with the padded boards small but definite shearing forces must be set up, and it appeared just possible that these give rise to cues which could be used by the subject to detect motion. There was one further uncertainty. As the stretcher oscillated a small change was observed in the level of the water on the two sides of the bath and there must have been correspondingly some currents

in the water below the surface. Introspections by the subjects did not suggest that this consideration was important except when some part of the body was very close to the surface. Nevertheless, there appeared to be a modification of technique worth investigation that would reduce both sources of error. By the immersion of the person not in water but in a viscous fluid the problem of trimming could be reduced, and the currents largely abolished. Accordingly, some observations were made with the bath filled with an industrial form of 'Polycell' cellulose paste. Four sets of results were obtained with the one subject used. In one of the four experiments the paste was thickened further by the addition of substantial

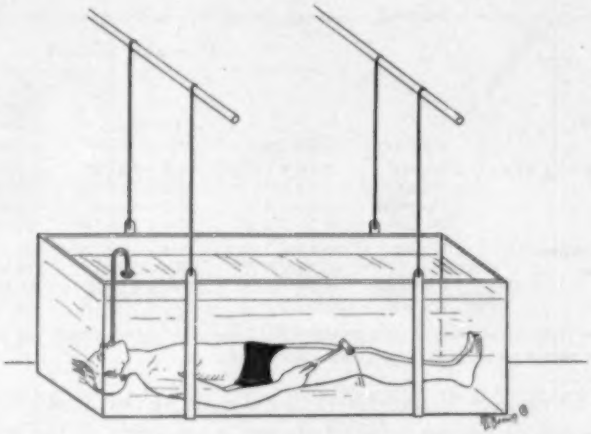

Fig. 2. Subject in rectangular tank. The padded boards at the two ends which prevent the subject floating to the surface are not shown.

quantities of sawdust. None of these procedures caused an increment of threshold. The mean value lying supine was $7{\cdot}1 \pm 2{\cdot}8$ cm/sec^2 and $4{\cdot}3 \pm 0{\cdot}7$ cm/sec^2 lying prone.

Effect of spinal injuries

The results obtained in normal subjects on the stretcher may be compared with those obtained with twelve patients suffering from high spinal lesions. The patients were tested at the Spinal Injuries Unit, Stoke Mandeville, and were aged 18 to 47. In each the spinal injury had resulted in a complete spinal transection, as far as could be determined clinically. In eight the transection was in the cervical region, in the others it was in one of the upper three thoracic segments; all the tests were in the supine position. For oscillations in the long axis of the stretcher the threshold

was $7{\cdot}9 \pm 2{\cdot}5$ cm/sec^2; for transverse movements the threshold was $3{\cdot}5 \pm 1{\cdot}9$ cm/sec^2. Six normal subjects tested at the same time gave values of $5{\cdot}3 \pm 3{\cdot}5$ cm/sec^2 for longitudinal movements and $3{\cdot}8 \pm 1{\cdot}9$ cm/sec^2 for transverse movements. These values are higher than the control data of Table 2, a difference which cannot with certainty be explained. The normal subjects were, however, hospital workers who were tested at one session only and had not had previous experience of sensory investigation. The values obtained from the spinal patients compare favourably with most of the figures reported by others from normal people (Table 1).

DISCUSSION

The present work appears to establish the role of the vestibular apparatus in the perception of linear movement. High values for threshold were found when the eighth nerves were damaged, normal or nearly normal values under conditions in which the importance of somatic sensibility was reduced. Other work already reported (Walsh, 1960) has dealt with the sensibility to linear motion after unilateral labyrinthectomy and forms additional evidence as to the role of the vestibular apparatus.

It is to be expected that a structure sensitive to linear acceleration will be sensitive also to tilting. A continued discharge during tilt will be expected, however, only if the endings show no adaptation and under certain circumstances receptors, other than those of the vestibular apparatus, play an important role in the perception of altered position (Garten, 1920). In the thornback ray (Lowenstein & Roberts, 1949) and in the cat (Adrian, 1943) persistent discharges in the vestibular system occur in response to tilt. A similar situation probably exists in the human being, for in response to tilting there is a persistent although small counter-rotation of the eyes (Woellner & Graybiel, 1959).

Linear motion may at times give rise to the sense of movement; on the other hand, motion on a parallel swing may give rise to a false sense of tilting (Jongkees & Groen, 1946). This type of confusion seems to be rare except with man-made contrivances which subject the person to linear acceleration of longer duration than would occur under natural circumstances. Rotation on a roundabout (or human centrifuge) produces a centrifugal acceleration that lasts as long as the platform turns. This acceleration gives rise, not to the sense of flying away in its direction, but to a sense of tilting. Under normal circumstances the body is not subjected to long-lasting linear accelerations, and it is possible that the potential ambiguity is usually resolved by systems that separate long-lasting from short-lasting signals. The general problems of dual sensitivity in sensory systems have been lucidly discussed by Cohen (1960).

Both Smith (1956) and Engström & Wersäll (1958) have demonstrated the existence of two types of sensory cell in the maculae of the otoliths; and on functional grounds a duplicity theory postulating systems with different time constants seems almost inescapable. A clearer definition of the conditions under which the different mechanisms come into play could be of some importance for the physiology of space travel.

SUMMARY

1. Thresholds have been determined for the perception of linear motion.

2. On a parallel swing the sensibility was found to be normal in patients with high spinal lesions, and in normal people during total immersion in water or paste. On the other hand, the thresholds were elevated in children with bilateral lesions of the eighth nerve system.

3. It is concluded that under the conditions of the experiments the sensitivity depends primarily on the vestibular apparatus.

The author is indebted to L. E. Heath, Esq., Headmaster of St Giles School, Colinton, and to the parents of the children for permission to conduct the tests on pupils at the school; and to Dr L. Guttmann and the staff of the Spinal Injuries Unit for facilitating the investigation of paraplegic subjects. Technical assistance was given by Mr G. Wright. Polycell Products Ltd. kindly made a gift of a substantial quantity of material. The figures were prepared in the Department of Medical Illustration.

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THE QUANTAL NATURE OF TRANSMISSION AND SPONTANEOUS MINIATURE POTENTIALS AT THE CRAYFISH NEUROMUSCULAR JUNCTION

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Of all the synapses that have been studied the physiology of neuromuscular transmission in vertebrates is most advanced, mainly as a result of the more recent microphysiological work by del Castillo, Fatt and Katz (Katz, 1958). The chemical nature of the transmission process having been established earlier, they found that transmission occurs by the simultaneous release of many packets or quanta of acetylcholine (ACh) from the motor nerve terminals. These quanta, each of which consists of many molecules of transmitter, cause in the post-synaptic membrane the end-plate potential. This sets up conducted impulses in twitch muscle fibres. Single quanta are also released spontaneously in a random sequence in 'resting' muscle.

In the crayfish and other crustacea the excitation process differs from that in vertebrates in many respects. The motor innervation is widely distributed over the muscle-fibre surface (van Harreveld, 1939; Wiersma, 1941). In many muscles the graded junctional potentials only rarely cause conducted impulses (Wiersma, 1941; Katz & Kuffler, 1946). Accordingly, much of the normal contractile activity in individual muscle fibres is graded. Further, the transmitter is different from that in vertebrates and most crustacean muscles also have an inhibitory innervation. In the face of these differences it was of interest to see if any evidence could be found that the mechanism of transmitter release in crayfish was similar, i.e. whether it also occurred in quantal steps.

This is the first of a series of papers in which we report on the quantal nature of transmission in the crayfish, how facilitation is linked with this quantal release of transmitter and how inhibition interacts with it. A preliminary note on spontaneous miniature potentials has already appeared (Dudel & Orkand, 1960).

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METHODS

Almost all experiments in the current studies were done on the abductor muscle of the dactyl (corresponding to the opener of the claw) in the 1st or 2nd walking leg of the crayfish *Orconectes virilis* (Steinhilberi). The muscle is relatively thin, consisting of several layers of short fibres which originate on the exoskeleton and are inserted on a central tendon. Most of the fibres which were used were 200–300 μ in diameter and about 2–3 mm long in the relatively small animals. The adductor of the dactyl was removed, leaving the flat inner surface of the abductor exposed. The intact exoskeleton around the muscle formed a natural chamber with a volume of not more than 0·1 ml. The leg was placed in a Lucite chamber, with the dactyl and the carpopodite fixed (Fig. 1). The inhibitory and excitatory axons to this muscle are contained in separate bundles which were exposed in the meropodite. The bundles were kept submerged in the second compartment of the chamber and were stimulated with fluid electrodes. These consist of glass tubes with a small opening, large enough for a nerve bundle to go through, connected to a syringe by fine tubing. Various lengths of nerve can be pulled up into the tube, together with physiological solution. The stimulus is then applied between the inside and outside of the glass tube.

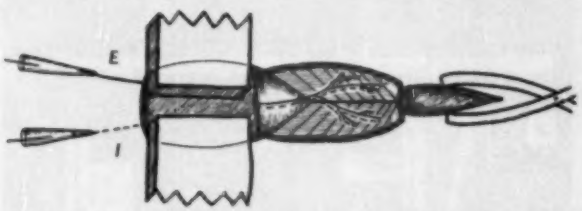

Fig. 1. Scheme of the preparation of the abductor of the dactyl in the crayfish, viewed from above. Adductor muscle removed. *E* = excitatory axon, *I* = inhibitory axon.

The composition of van Harreveld's solution (1936) is (mM) NaCl 195, KCl 5·4, $CaCl_2$ 13·5 and $MgCl_2$ 2·6. The pH was kept near 7·5 with 10 mM tris maleate buffer. Most experiments were done at 21–23° C. The muscle fibres were kept covered by physiological solution and the fluid was periodically replaced by adding a few drops of fresh solution. Alternatively a constant stream of fluid was kept flowing past the muscle fibres.

The recording system consisted of conventional DC and AC amplifiers. The micropipettes were generally filled with 3M-KCl and had resistances of 7–20 MΩ. Their tip diameters were 1 μ or smaller. For exploration of the potentials along the fibre surface, larger pipettes (1–5 MΩ) were sometimes used, filled with saturated NaCl solution and having tips of up to several micra diameter.

Muscle fibres were viewed under dark-field illumination, which enabled one to see the course of nerve branches. The finer ramifications near the junctions were not visible. The excitatory and inhibitory axons run together and also branch in unison innervating fibres at multiple spots (van Harreveld, 1939; Fatt & Katz, 1953).

RESULTS

Spontaneous miniature potentials

If one inserts a micro-electrode into the abductor muscle fibres anywhere along their course, one generally observes, in the absence of nerve stimulation, small potential changes of variable size, as shown in Fig. 2*A*. They

usually occur at frequencies around 1/sec but in some preparations they are seen only once every few seconds. Rarely does the frequency exceed 5/sec. On analogy with similar potentials in vertebrate muscle (Fatt & Katz, 1952) they will be called spontaneous miniature potentials (Dudel & Orkand, 1960). In Fig. 2*C* the time course of one miniature potential is

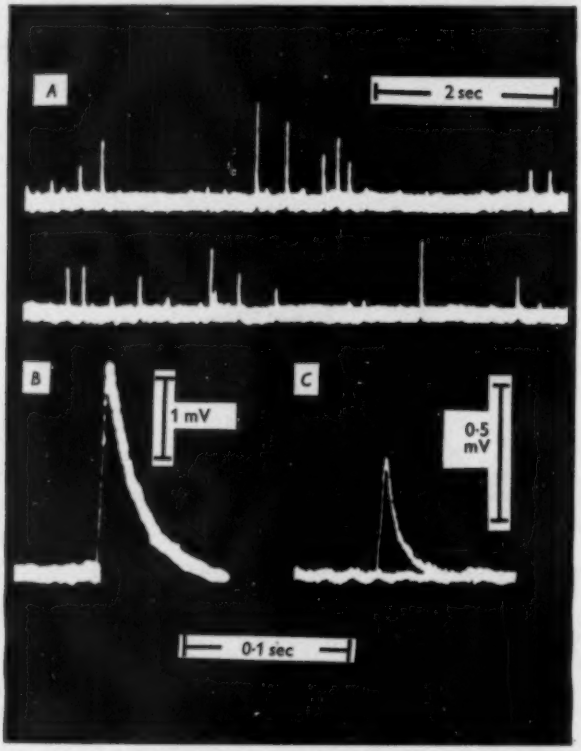

Fig. 2. A and *C*: Spontaneous miniature potentials recorded with an intracellular electrode from the abductor of the dactyl. Resting potential 75 mV. *B*: Excitatory junctional potential (e.j.p.), set up by single motor nerve impulse; note similar time course to spontaneous potential in *C*. Voltage calibration the same in *A* and *C*. Time calibration same in *B* and *C*.

displayed on a fast sweep speed for comparison with an excitatory junctional potential (e.j.p.) in Fig. 2*B*, set up by stimulation of the motor axon. The only difference is in peak height, the miniature potential being about 0·43 mV, whereas the e.j.p. reaches 2·5 mV.

In vertebrates the miniature potentials are confined to the end-plate regions of fibres, but in the abductor of the dactylopodite they are recorded

everywhere, as are the e.j.p.s. E.j.p.s can be recorded wherever microelectrodes are inserted into fibres, because nerve–muscle junctions occur at multiple sites over the muscle fibre surface (Wiersma, 1941; Fatt & Katz, 1953). It will be shown below that spontaneous miniature potentials originate at the same areas. Further, the spatial decrement of potentials along the muscle fibres is relatively small, so that an impressed potential change at one end does not decline by more than 40% over the entire length. Therefore, local junctional potentials are detected by an intracellular lead everywhere within muscle fibres with relatively little attenuation.

In many fibres spontaneous miniature potentials were not seen and in others they barely exceeded the noise level (20–30 μV) of our DC amplifying system. It appeared that those were large fibres, in which the input resistance, measured by a voltage drop accompanying intracellularly applied current pulses, was relatively low. The input resistance had to be between 0·2 and 0·5 MΩ for miniature potentials above 100 μV to be recorded. Accordingly, the potentials are larger in small-diameter fibres, as is also seen in vertebrates (Katz & Thesleff, 1957). This also applies to the e.j.p.s set up by motor nerve stimulation.

The following observations show the analogy between our spontaneous miniature potentials and those obtained in vertebrate junctions. (1) As in end-plates, spontaneous potentials appear at irregular intervals in a random sequence. Figure 3 gives a distribution of intervals of 240 miniature potentials. The broken line is drawn according to the equation

$$n = N\frac{\Delta t}{T}\exp\frac{-t}{T},$$

where n = the number of observed potentials at any interval,
N = number of observations,
T = mean interval,
Δt = duration of the grouping in the histogram (0·2 sec) and
t = observed interval between successive potentials (Fatt & Katz, 1952).

The fit of the observations with the curve means that there is no obvious interaction between single spontaneous potentials. (2) A little understood feature of vertebrate miniature potentials is the sensitivity with which they increase in frequency with raised osmotic pressure. This also applies to the miniature potentials in crayfish. For instance, doubling the osmotic pressure by adding sucrose has resulted in as much as a sevenfold increase in rate. (3) In vertebrate junctions it was shown that the membrane potential of the motor nerve terminals controls the average miniature frequency. This observation is important because it is probably related to

the conversion of motor nerve impulses to transmitter release. Nerve terminals in the crayfish preparation could not be depolarized by applied current. We were able, however, to depolarize the endings by excess K^+ and to get increased rates of miniature release (Liley, 1956*c*). For instance, doubling the K^+ concentrations resulted in a muscle depolarization of about 7 mV and doubled the spontaneous discharge rate. The size of the spontaneous potentials was not appreciably changed nor was the membrane resistance affected. An even larger depolarization of the muscle

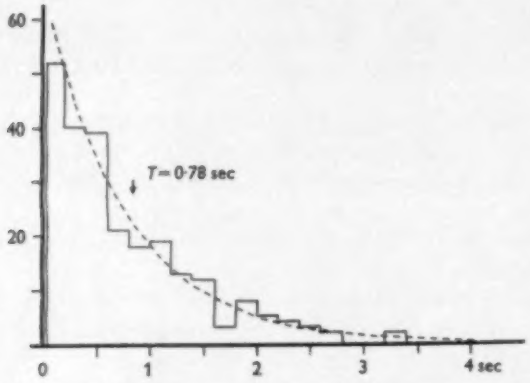

Fig. 3. Distribution of intervals between 240 spontaneous miniature potentials. Interrupted line is distribution expected for random sequence (see text). *T* is the mean interval between potentials.

membrane by applied current did not change spontaneous discharge rates, and thus the potassium effect could not have resulted solely from the change in muscle membrane potential. It is therefore thought that the effect was brought about by the depolarization of nerve terminals by excess K^+. (4) Another point that might relate membrane changes in the motor nerve with the discharge rate of the spontaneous miniature potentials is the transient change in their frequency following trains of excitatory potentials (cf. Dudel & Kuffler, 1961). This again resembles the behaviour of vertebrate junctions.

Fatt & Katz (1952) and del Castillo & Katz (1954) showed that in frogs miniature potentials were not set up by spontaneous conducted impulses in the motor axon. This possibility was excluded in the crayfish because the miniature potentials were not abolished by excess Ca^{2+} even if increased tenfold, or by application of β-guanidopropionic acid 10^{-4} g/ml. (Kuffler, 1960). Such treatment abolished nerve conduction and no e.j.p.s appeared with nerve stimulation. Further, extracellular recording with

micro-electrodes showed that during a single miniature potential only one active synaptic area is involved, whereas a nerve impulse activates many junctional areas (see below, Fig. 6).

There remains the unlikely possibility that spontaneous miniature potentials arise as a result of spontaneous impulse-like activity in single terminals without invasion of the axons. This also was excluded, because spontaneous miniature potentials, interspersed between e.j.p.s, did not show facilitation (Dudel & Kuffler, 1961).

Extracellular records from single junctional areas

The question now is whether the e.j.p.s set up by motor nerve impulses are built up of units that resemble the spontaneous miniature potentials. To answer this is made difficult because the innervation of the abductor fibres is distributed widely in multiple spots over the muscle surface. Intracellular leads record all the potential changes, with some attenuation, which take place across the surface membrane of the short muscle fibres. They sum or integrate the many junctional potentials and correspondingly complicate the analysis. Our question would, therefore, be better answered by recording exclusively from a single junction formed by the synaptic contact of one terminal branch.

Del Castillo & Katz (1956*a*) have shown that with extracellular micro-electrodes one can record from one 'active' spot within the vertebrate end-plate. They have discussed the recording conditions extensively and we have used this method in the crayfish. Briefly, the situation is as follows. The released transmitter causes a synaptic current to flow through the junctional membrane. The field in the low-resistance external medium is very circumscribed and only within a few micra of the small junctional membrane area will the synaptic current be dense enough to cause an appreciable voltage drop. If placed close to the junctional patch a sufficiently small extracellular electrode will record this voltage drop and thus one can obtain a relative measure of the synaptic current at an individual junctional area.

The main problem is to find such a circumscribed region. If one places a capillary electrode (0·5–2 μ opening) on the muscle surface, no e.j.p.s are seen as a rule. If, however, one conducts a tedious search along the fibre, making on the average 30–50 contacts, one usually finds a spot where potentials appear with nerve stimulation. These spots are sharply localized and the potentials disappear with a sideways movement of the electrode tip of several micra.

In Fig. 4 the motor nerve was stimulated at 5/sec. The upper line shows the e.j.p.s recorded intracellularly. The lower line presents the simultaneous e.j.p.s measured extracellularly at a single junctional area. The

e.j.p.s from the fibre interior are relatively large and show fluctuations in height that are usual at such low frequencies. The intracellular potentials are positive, whereas the surface micro-electrode records negative potentials. The most interesting aspect is the relationship of potential fluctuations in the two records. Under the extracellular micro-electrode the single synaptic area did not give any response with one of the nerve stimuli (arrow). At that time, however, the intracellular electrode happened to record the largest e.j.p. in the series. With this particular nerve stimulus many of the junctions, but not the one under the extracellular lead, must have been activated. Also, in the other records there is no obvious correlation between the intracellular potential size and the extracellular one. More detail of time relations is shown in Fig. 5 where the sweep speed is rapid.

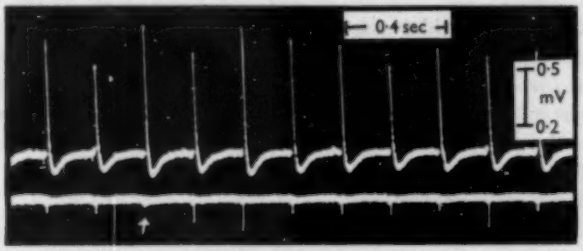

Fig. 4. Upper line: intracellular recording of e.j.p.s at stimulation rate of 5/sec. *Resting potential 82 mV. Lower line:* simultaneous records from a single junctional area with an extracellular microelectrode. Arrow marks failure of transmission in single junction; note independence of fluctuations in intra- and extracellular records. *0·2 mV refers to amplification in lower beam. Diphasic component of* intracellular records is due to AC amplifier.

Five records, taken consecutively during stimulation at a rate of 2/sec, are superimposed. The intracellular e.j.p. sizes in the upper record fluctuated very little, but in comparison the potentials at the single junctional area varied over a wide range. Two nerve impulses failed altogether to set up an extracellular e.j.p. Preceding the extracellular junctional potentials by about 1 msec, there occurred regularly another phasic potential which represents the nerve impulse (arrow) arriving in the vicinity of the junction. A neuromuscular delay, thus measured, of about 1 msec was seen regularly. The intra- and extracellular e.j.p.s had practically the same latent period; the duration of the extracellular potentials, however, did not last much longer than the rising phase of the intracellular e.j.p.s. Apparently the 'active' phase, during which the transmitter causes synaptic current to flow, is largely confined to the rising phase of the e.j.p. Following this period, the potential decline of the intracellular e.j.p.s is passive, i.e. determined by the muscle time constant. The current that

flows passively is distributed over the whole membrane area. The membrane current density is therefore low and the resulting extracellular potential drop is too small to be measured.

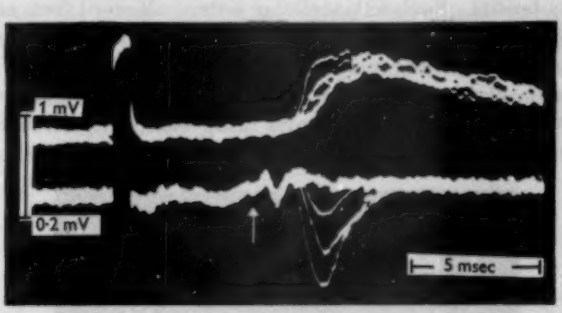

Fig. 5. Upper line: intracellular recording of e.j.p.s at stimulation rate of 2/sec, five sweeps superimposed. Lower line: simultaneous extracellular e.j.p.s from a single junctional area recorded with micro-electrode. Arrow marks beginning of motor nerve impulse near junctional area. Latent period of intra- and extracellular e.j.p.s is same but time course of extracellular e.j.p.s is shorter and their range of fluctuation is much greater. Two of the five stimuli fail to set up e.j.p.s in extracellular record.

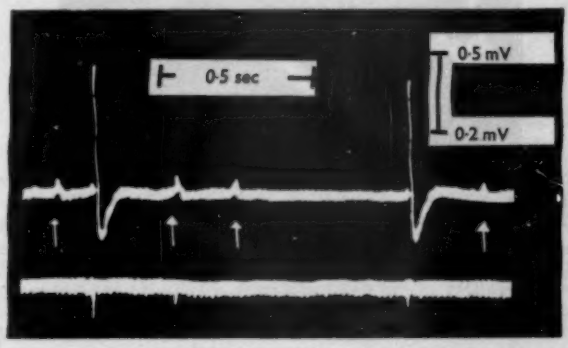

Fig. 6. Motor nerve stimulated at 1/sec. Upper line: intracellular record of two e.j.p.s and of four spontaneous miniature potentials (arrows). Lower line: simultaneous extracellular record from a single junctional area. Only one spontaneous potential occurs (simultaneous with second arrow). It is of the same amplitude as the second e.j.p. set up by a nerve impulse. 0·2 mV refers to lower beam amplification.

In addition to e.j.p.s one could record spontaneous potentials if the extracellular electrode was placed at an 'active' spot. An example is given in Fig. 6. The nerve was stimulated at 1/sec and two extra- and intracellular e.j.p.s are illustrated. Intracellularly, four spontaneous

miniature potentials are also seen (arrows). The second one is associated with an extracellular spontaneous potential. When such a potential occurred it was always accompanied by an intracellular spontaneous miniature potential. Such extracellular potentials were seen only about once a minute, as compared with intracellular ones which occurred about once a second. They were found exclusively at the active spots where the localized extracellular e.j.p.s were recorded. With a small sideways shift of the electrode, both potential types disappeared. Spontaneous extracellular potentials had a size similar to that of the smallest e.j.p.s set up

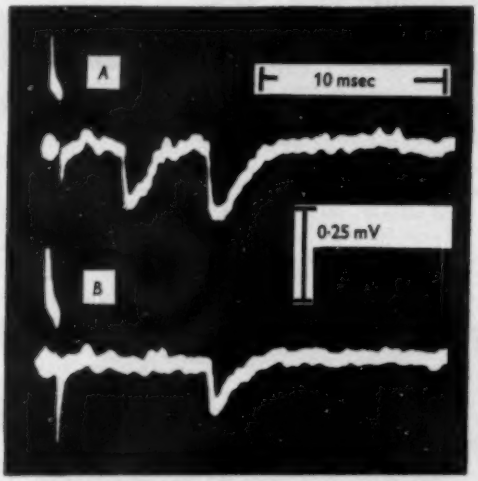

Fig. 7. Extracellular records from single junctional area during motor nerve stimulation. *A*. Second potential is e.j.p. set up by nerve impulse; first is a spontaneous miniature potential. *B*. e.j.p. set up by motor nerve impulse as in *A*. Note similar size and time course of e.j.p.s and of the spontaneous potential.

by nerve stimulation and recorded at the same spot. An example is contained in Fig. 6 (second e.j.p.). Not only sizes, but also the time courses of the smallest extracellular e.j.p.s and of spontaneous potentials, were practically identical. In Fig. 7*A* a spontaneous potential, by chance, fell just before an e.j.p. taken at a fast sweep. The two e.j.p.s (Fig. 7*A* and *B*) set up by nerve impulses indicate the range of fluctuation of this response.

One can conclude that the intra- and extracellular spontaneous potentials represent the same phenomenon. Like the e.j.p.s, they arise at neuromuscular junctional areas. The fact that many more intracellular than extracellular spontaneous potentials are observed means that spontaneous potentials arise at numerous terminal regions. The ratio of about

1:60 of the frequency of extracellular to intracellular spontaneous potentials may be a rough measure of the number of synaptic contact areas in a muscle fibre. The striking similarity of the spontaneous extracellular potential and the smallest e.j.p. set up by nerve impulses leads one to suspect that, as in the frog end-plate, the spontaneous potentials represent the same units as those released normally in great numbers upon nerve stimulation (see later).

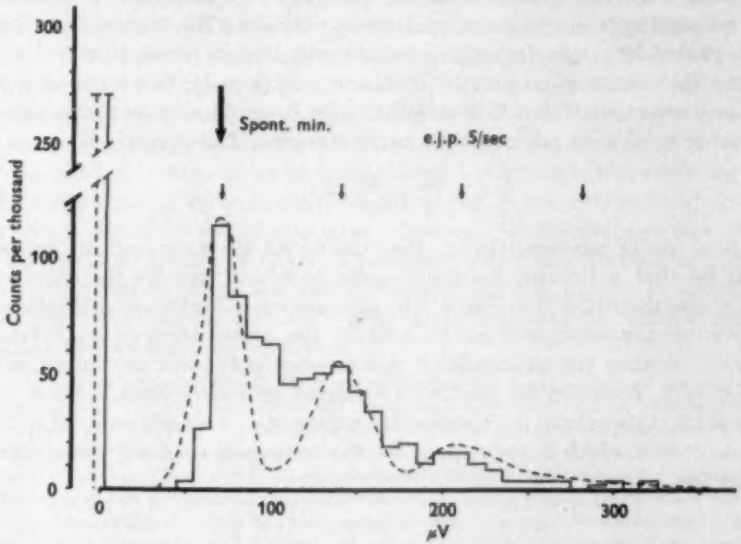

Fig. 8. Histogram of size distribution of extracellularly recorded e.j.p.s from a single junctional area. Ordinate scaled to 1000 counts. There are 270 zero potentials, i.e. failures of transmission. Broken line is drawn according to Poisson's theorem for a quantum size of $E_1 = 70$ μV with a standard deviation $\sigma_1 = 12$ μV. The quantum content per stimulus is $m = 1{\cdot}3$. Small arrows indicate the multiples of the unit size. Big arrow gives the average size of the spontaneous miniature potentials.

Statistical treatment of extracellular e.j.p.s from single synaptic areas

The occurrence of spontaneous miniature potentials and the striking fluctuations in the size of the e.j.p.s recorded extracellularly (Figs. 4 and 5) suggest an analogy with the situation at the vertebrate neuromuscular junction, i.e. that the crustacean e.j.p. is also built up of units representing quantal release of transmitter. In order to test the applicability of this idea more closely, a statistical analysis of the type used by del Castillo & Katz (1954), Boyd & Martin (1956) and Liley (1956*b*) was needed. We recorded from an extracellular junctional area the responses to 1000 nerve

stimuli given at a rate of 5/sec. The graph of Fig. 8 is a histogram of the recorded e.j.p. sizes. The distribution does not fall symmetrically around a mean but has a complicated form. It contains 270 misses, i.e. occasions when an impulse failed to set up any extracellular junctional potentials.

If the potentials are made up of unit responses one expects that Poisson's theorem should describe the distribution. In the context of the present problem this theorem applies if there exists a very large pool of possibly reacting units and if the probability that any one particular unit should be released on a single nerve stimulus is very low. The theorem predicts the probability n_x/n (n number of stimuli) that a nerve stimulus will cause the release of no unit ($x = 0$), one unit ($x = 1$), two units ($x = 2$) or any other multiple. It states that n_x/n depends only on the average number m of units released by a nerve stimulus. The equation is:

$$\frac{n_x}{n} = \frac{m^x}{x!}\,e^{-m} \tag{1}$$

A first test of our assumption, that the e.j.p.s are composed of quanta, will be that a Poisson distribution can be found that fits the observed e.j.p. size distribution in Fig. 8. The information gained from spontaneous miniature potentials will not be used for the construction of the Poisson curve, because the extracellular spontaneous potentials are infrequent. They will, however, be used afterwards as an independent control. If equation (1) is written for consecutive values of x, a simple series of equations results which is convenient for the construction of a Poisson curve that fits:

$$n_0 = n\,e^{-m}, \tag{2a}$$
$$n_1 = m\,n_0, \tag{2b}$$
$$n_2 = (\tfrac{1}{2}m)\,n_1, \tag{2c}$$
$$n_3 = (\tfrac{1}{3}m)\,n_2. \tag{2d}$$

It is obvious from this series that if n_0 is known, m can be calculated from equation (2a), and then all the values n_1, n_2, n_3, ... follow.

Experimentally n_0 can be found by merely counting the number of failures. Thus this value is a convenient starting point for the construction of a Poisson curve which fits the histogram of Fig. 8. According to equation (2a) for $n_0/n = 270/1000$ we calculate an average number of units released per stimulus of $m = 1{\cdot}3$. And the equations (2) further predict the number n_1, n_2, n_3, ... of potentials of single and multiple quantum size. These numbers now have to be converted into unit potentials. The size of the average unit potential E_1 can be obtained from the relation

$$E_1 = \frac{\bar{E}}{m}, \tag{3}$$

where $\bar{E}$ is the average size of the extracellular e.j.p. which can be measured.

For the experiment of Fig. 8, E_1 was 67 μV. The unit quantum is not strictly of the same size each time it is recorded. E_1 is an average unit potential with a statistical fluctuation around a mean value. For instance, the number of single units n_1 represents the area under a Gaussian curve (normal distribution) of a certain variance σ_1 around the unit size E_1. As a consequence the appropriate Poisson curve for Fig. 8 is obtained by finding σ_1, by trial and error, and adding the Gaussian curves with the areas n_1, n_2, n_3, ... around the mean values E_1, E_2, E_3, ... The procedure is illustrated in Boyd & Martin (1956). It is seen in Fig. 8 that the general form of the Poisson curve agrees with the experimental values.

A control and confirmation of the validity of calculating unit sizes comes from measuring the average size of the spontaneous potentials. The spontaneous miniatures should be of unit size, because it is very improbable that two or more units should be released spontaneously at the same time. During the experiment of Fig. 8, ten extracellular spontaneous miniature potentials were observed. Their average size was 71 μV with a variance of 10 μV. This agrees very well with the size of the unit quantum E_1 calculated for the Poisson curve in Fig. 8. It can thus be concluded that the e.j.p.s are composed of units of the same average size as the spontaneous potentials.

In the above statistical treatments of the extracellular e.j.p.s we calculated the unit size from the relative number of failures and the average e.j.p. size. Then we compared this value with the average size of the spontaneous potentials. In the statistical papers on the vertebrate end-plate the converse procedure was used by del Castillo & Katz (1954), Boyd & Martin (1956) and Liley (1956*a*). They determined first the average spontaneous potential size and used it as the unit quantum for the construction of a Poisson curve, which had to fit the observed distribution of end-plate potential sizes. This approach was not used in this study because the extracellular spontaneous potentials are too infrequent in the crayfish junctions for the average size of spontaneous potentials to be accurately determined.

Equation (2*a*) provides an easy way of determining quantum size and quantum content in different junctional areas. Some examples are given in Table 1; the unit size E_1 has been calculated there from the relative number of failures n_0/n and the average potential $\bar{E}$. As expected, in all the samples the values for E_1 agree with the values found for the average spontaneous potential. These examples add further weight to the above conclusion that the e.j.p.s are indeed quantal responses. Further supporting examples, as in Table 1, are given in the following papers.

DISCUSSION

The following picture of crayfish neuromuscular transmission has emerged from the present study. There are discrete junctional areas which occupy a small portion of the muscle fibre surface, as seen from the fact that a prolonged search is necessary to locate them. In these spots synaptic

currents flow after nerve stimulation and it is these currents that have been shown to be quantal in nature. We assume that the quanta of current represent quanta, or units, of transmitter released from nerve terminals. At the same spots there occur spontaneous miniature potentials which are of unit quantum size. They are not released by spontaneous impulse activity in the axons or in the nerve terminals (see also Dudel & Kuffler, 1961, p. 536).

In all aspects covered by this study there was a rather complete analogy between mechanisms of transmission in vertebrates (del Castillo & Katz, 1956*b*) and in crayfish. A quantitative difference in the transmission process is the much lower probability of release of quanta in individual junctional areas of the crayfish. Our preparation in a 'normal' solution works like the nerve–muscle junction of twitch fibres in the frog, where

TABLE 1

Expt. no.	Stimulus frequency	n	m	$\bar{E}(\mu V)$	$E_1(\mu V)$	$E_s(\mu V)$
1	5/sec	121	2·2	155	70	70
2	5/sec	961	1·32	85	65	69
3	2/sec	243	0·27	18	66	65
4	1/sec	115	0·31	15	49	48

n = number of stimuli; m = average quantum content of extracellular e.j.p. determined from $m = \log_e n/n_0$; n_0 = number of transmission failures; $\bar{E}$ = average size of extracellular e.j.p.; E_1 = size of quantum determined from $E_1 = \bar{E}/m$; E_s = average size of extracellular spontaneous potentials.

the probability of release was depressed by excess Mg^{2+} and/or decreased Ca^{2+} (del Castillo & Katz, 1954). Spontaneous potentials of low frequency were seen by Burke (1957) in the frog's slow muscle fibres.

Another interesting difference is the shorter duration of synaptic current flow in the crayfish. The current barely outlasts the e.j.p. rising phase, whereas in the frog there is considerable current flow during the first half of the falling phase (del Castillo & Katz, 1956*a*; Liley 1956*a*; Takeuchi & Takeuchi, 1959).

A weak point in our line of conclusions about the transmission mechanism lies in our having measured quantal synaptic currents only. We interpret these quanta of currents as caused by packets of transmitter. We do not have direct evidence for this view. There is a possibility that the transmitter itself is not released in packets but triggers a quantal response in the post-synaptic membrane. We consider this possibility to be very unlikely, mainly because of the complete analogy that exists with the vertebrate junction. There this alternative was excluded because the transmitter is known. The size of the quanta could be graded by changing the amount of transmitter reaching the post-synaptic receptor sites by the use of applied ACh, curare, or of inhibitors of cholinesterase. These sub-

stances, however, did not affect the number of released units. In our preparation these experiments cannot be done because the transmitter is not known.

The extension of the quantal release mechanism of transmission from frog and mammalian neuromuscular junctions (del Castillo & Katz, 1956*b*); Boyd & Martin, 1956; Liley, 1956*a*) to crayfish makes one suspect that this system may be a much more general feature of synapses. In particular, the quantal mechanism is not confined to the cholinergic systems because in crayfish junctions ACh is not a transmitter. In addition, the quantal mechanism does not seem to be confined to a specific anatomical synaptic structure. The mammalian neuromuscular junction has a compact terminal configuration, being much more confined than in the frog, where the nerve splits up into a fine terminal arborization which may extend over several hundred micra. (For a survey, see Couteau & Taxi, 1952.) The innervation in the frog's slow fibres is also spread over most of the surface, as in the crayfish (Kuffler & Vaughan Williams, 1953), and spontaneous miniature potentials have been demonstrated there (Burke, 1957). In the crayfish abductor of the dactyl the widespread innervation formed by one motor fibre may be regarded as an extended 'end-plate'. The fine detail of the junctions could not be resolved by the light microscope. Another different feature of the muscles is the inhibitory innervation which, as far as can be seen, runs together with the motor fibre (Biedermann, 1887; Mangold, 1905; Hoffmann, 1914; D'Ancona, 1923; Tiegs, 1924; van Harreveld, 1939).

The statistical treatment of the excitatory potential, showing its quantal composition, was found useful in this study for linking the spontaneously released miniature potentials to excitatory transmission. This quantal analysis of the e.j.p. gains further significance in the following papers; it was found that the unit sizes remain constant during drastic changes of the e.j.p. brought about by the physiological mechanisms of facilitation and inhibition.

SUMMARY

1. The abductor of the dactylopodite in the crayfish is supplied by only one motor axon. Over the surface of each muscle fibre there are distributed numerous individual junctional areas. Intracellular electrodes record practically all excitatory junctional potentials (e.j.p.s) of a muscle fibre. Extracellular capillary electrodes record, selectively, the strictly localized potentials of individual junctional areas.

2. The e.j.p.s at individual junctional areas are 'quantal'. They are composed of discrete units released with a certain probability by a motor nerve impulse. This conclusion is based mainly on the finding that the distribution of sizes of e.j.p.s can be described by Poisson's theorem. The

probability of release of quanta at individual junctional areas is low, and frequently a nerve impulse fails to release a single quantum. The e.j.p. of the whole muscle fibre is thus the sum of all the quantal contributions from distributed single junctional areas.

3. In addition to e.j.p.s, spontaneous miniature potentials can be recorded. They arise at the same individual areas and have the same size and time course as the units released by the nerve impulse. Spontaneous miniature potentials recorded intracellularly recur in a random sequence at rates around 1/sec. Recorded extracellularly at single junctional areas they are seen about once a minute. Their frequency but not their size is influenced by repetitive motor nerve stimulation, raised osmotic pressure and moderate depolarization by excess K^+. Spontaneous miniature potentials are not due to spontaneous nerve impulses, because they are not affected when nerve conduction is blocked by high Ca^{2+} or by β-guanidobutyric acid.

4. The quantal nature of transmission in the crayfish is similar to that at vertebrate neuromuscular junctions. It is concluded that the quantal e.j.p.s are set up by the release of packets of transmitter from motor nerve endings.

We wish to thank Mr Richard Orkand who participated in some of the experiments. To Dr E. J. Furshpan and Dr David Potter we are indebted for continued helpful discussions and to Mr R. B. Bosler for unfailing technical assistance. The study was supported by the U.S. Public Health Service.

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MECHANISM OF FACILITATION AT THE CRAYFISH NEUROMUSCULAR JUNCTION

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The term facilitation frequently covers a variety of processes which lead to an increased response, including temporal and spatial summation, recruitment, or removal of inhibition. A more restricted definition will be used here; we shall speak of 'facilitation' if a nerve impulse in a presynaptic fibre sets up an increased post-synaptic potential due to preceding impulses in the same nerve fibre. The phenomenon has been described in many synapses and it is well known in the crustacean neuromuscular junction (van Harreveld & Wiersma, 1936; Katz & Kuffler, 1946).

Three mechanisms have frequently been used in accounting for facilitation. The first explanation assumes that not all terminal regions in a presynaptic axon are reached by impulses and that more complete invasion of branches is brought about by facilitation. The second possibility is an increased output of transmitter by an individual nerve terminal causing a bigger synaptic potential. Finally, facilitation could consist of an increased response of the post-synaptic membrane to the same amount of transmitter.

A rigorous study of all the above possibilities has been made in the vertebrate neuromuscular junction only, and evidence was found for an increase in the statistical probability of release of transmitter quanta (del Castillo & Katz, 1954*a*). In this paper we find the same to be true in the crayfish.

The methods used are similar to those described in the preceding paper (Dudel & Kuffler, 1961*a*).

RESULTS

An example of facilitation is given in Fig. 1*A*, where the motor nerve to the abductor of the dactyl was stimulated by nine impulses at a rate of 20/sec. The first excitatory junctional potential (e.j.p.) recorded by an intracellular electrode is much smaller than the subsequent ones. At a stimulation rate of 40/sec (Fig. 1*B*) the individual e.j.p.s increase even

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more and, in addition, there occurs summation because at this frequency e.j.p.s add to the falling phase of the preceding potentials.

Although this study does not deal specifically with inhibition, an example of facilitation of inhibitory junctional potentials (i.j.p.s) is shown in Fig. 1*C* and *D* at frequencies of 23 and 58/sec. The potentials are of longer duration and therefore summation is more pronounced in the i.j.p. series. In this case the inhibitory equilibrium level was about 3 mV more positive than the resting potential, so that inhibitory impulses depolarized toward that potential level. One must consider that in Fig. 1*D* the potentials are approaching close to the inhibitory equilibrium level and this fact will limit their size (Dudel & Kuffler, 1961*b*).

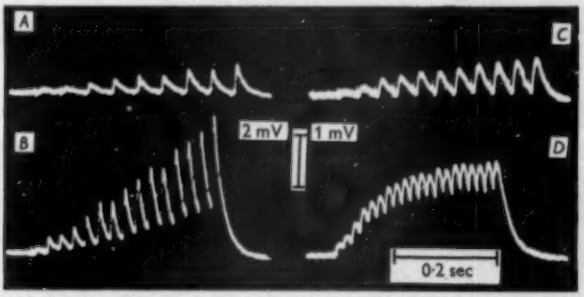

Fig. 1. Facilitation of excitatory (*A* and *B*) and inhibitory (*C* and *D*) junctional potentials. Intracellular recording, resting potential near 80 mV. *A* and *C*: stimulation rates 20 and 23/sec. *B* and *D*: stimulation rates 40 and 58/sec. Note increased amplification for the inhibitory potentials.

There are numerous other ways of demonstrating facilitation. For instance, trains of impulses, as in Fig. 1, can be repeated every few seconds and one sees that consecutive trains reach a greater potential peak. Or one can give a single conditioning train and test with a single e.j.p. afterwards. In such post-tetanic facilitation the test pulse may be facilitated for 20 sec or more, depending on the frequency of the preceding stimulation (*v. infra*, Fig. 6).

Facilitation at a single junctional area

In the previous paper (Dudel & Kuffler, 1961*a*) it was shown that neuromuscular transmission in the crayfish occurs in quantal steps. By analogy with the frog neuromuscular junction (del Castillo & Katz, 1954*a*), one might expect that facilitation should also involve this quantal mechanism, i.e. the e.j.p. increase is due to the release of a greater number of transmitter units. The unit size, however, should not change.

The most favourable conditions for analysing facilitation are attained

by recording, exclusively, from a single junctional area with an extracellular micro-electrode (Dudel & Kuffler, 1961*a*). At the same time one can register with an intracellular lead the distributed junctional activity which takes place along the whole muscle fibre surface. Figure 2 shows an example at two frequencies of excitatory stimulation. The upper lines are intracellular records, the lower ones extracellular. At 1/sec each nerve impulse sets up a relatively small positive e.j.p. as recorded intracellularly.

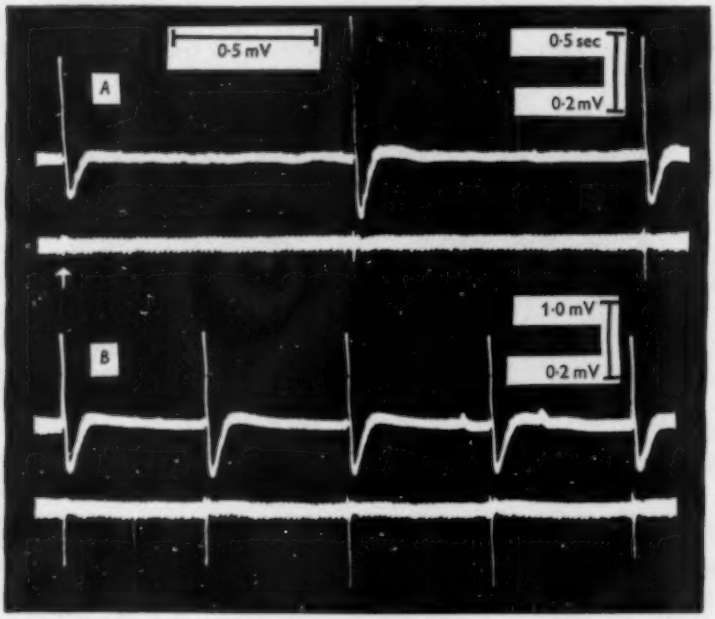

Fig. 2. Facilitation of intra- and extracellular e.j.p.s. In *A* and *B* the upper beam is an intracellular record, the lower beam is extracellular from a single junctional area. *A*: stimulation frequency 1/sec; arrow marks failure of transmission. *B*: stimulation rate 2/sec; note halving of amplification of intracellular beam. 'Positive after-potential' of the intracellular e.j.p.s is due to AC amplifier. 0·2 mV refers to lower beam.

The single junctional area at the extracellular electrode showed intermittent activity only, and once there was complete failure of transmission (arrow). At the higher frequency of 2/sec, the extracellular lead never failed to record activity, although the potential fluctuations were great; in comparison, the intracellular e.j.p. was reasonably steady and also much larger (note halved amplification). Figure 3 gives more detail on a fast sweep at repetition rates of 1 and 5/sec. Extracellularly, at 1/sec

there was one failure of transmission, while at 5/sec there was no failure and the potentials were larger and more uniform. The extracellular lead records the rapid time course of the negative e.j.p.s at one junctional spot, caused by the synaptic current entering this restricted area. The intracellular e.j.p.s are of opposite polarity and longer duration (for details of recording conditions, see Dudel & Kuffler, 1961*a*). The arrows indicate the start of the motor nerve impulse.

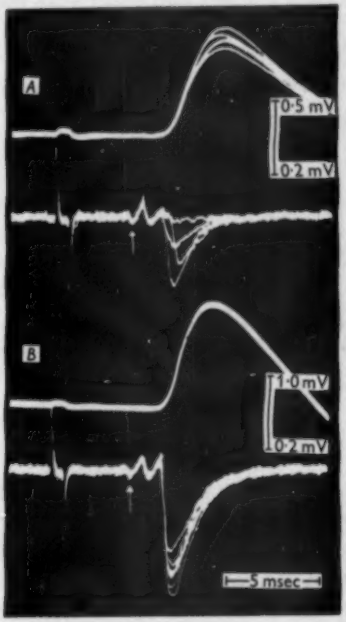

Fig. 3. Intra- and extracellular e.j.p.s, experiment of Fig. 2. *A*: four sweeps superimposed, stimulation rate 1/sec; one failure of transmission in extracellular record on lower beam. *B*: seven sweeps superimposed at stimulation rate of 5/sec; note decrease of fluctuation of intra- and extracellular e.j.p.s. Arrows mark motor nerve impulse.

Figures 2 and 3 have shown that at higher frequencies of stimulation there are fewer failures for transmitter to be released at individual junctions. Records like those of Fig. 3 are reminiscent of events at the frog neuromuscular junction in solution of high Mg content. There facilitation is known to depend on the number but not on the amplitude of released quanta (del Castillo & Katz, 1954*a*). In order to test this question statistical treatment was undertaken.

Statistical treatment

Figure 4 (upper part) presents a distribution of potential sizes recorded with an extracellular electrode at a single junctional area during 100 nerve stimuli at 1/sec. There were 67 misses, i.e. occasions when no release occurred. If the stimulation frequency was increased to 5/sec the curve (Fig. 4, lower part) was extended to the right, i.e. the junctional potential sizes with each stimulus increased and there were only ten misses.

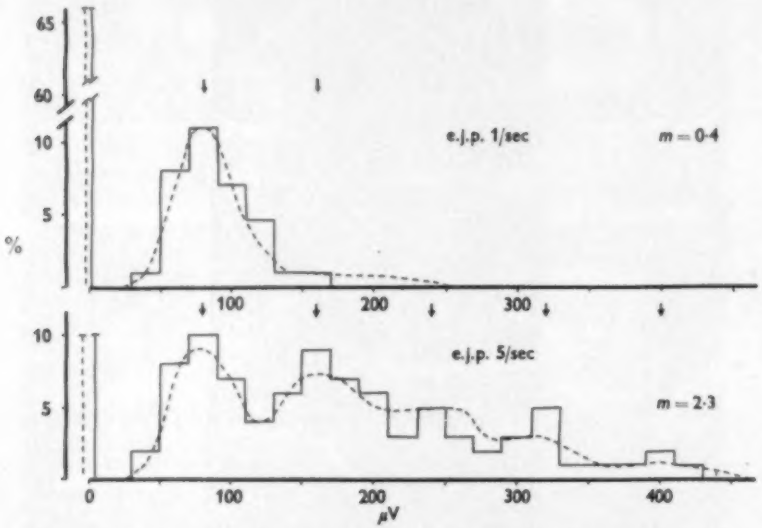

Fig. 4. Histograms of distribution of sizes of extracellularly recorded e.j.p.'s from a single junctional area. Abscissa: size of e.j.p.s. Ordinate: number of e.j.p.s counted, scaled to 100 stimuli. Stimulation frequency 1/sec in upper graph and 5/sec in lower graph; note decrease of transmission failures with facilitation from 66 to 10. Interrupted curves were calculated according to Poisson's theorem (see text). Unit size $E_1 = 80\ \mu V$ and its standard deviation $\sigma_1 = 20\ \mu V$ in both curves. Quantum content per stimulus m rises with facilitation from 0·4 to 2·3. Arrows mark multiples of quantum size.

To find the quantum content of the observed e.j.p.s at 1/sec one has to fit a Poisson curve to the histogram of Fig. 4. The Poisson theorem

$$\frac{n_x}{n} = \frac{m^x}{x!}\, e^{-m} \qquad (1)$$

predicts the relative number n_x of potentials x times the unit size, that will be counted if m is the average number of units released per stimulus.

The appropriate Poisson curve was determined for the e.j.p. size dis-

tribution at 1/sec by the procedure described in the preceding paper (Dudel & Kuffler, 1961*a*). It was found that the unit quantum size E_1 was 80 μV and the average quantum content of the e.j.p.s was very low ($m = 0{\cdot}4$). Therefore, most of the observed potentials must have been unit quanta. Accordingly, if the failures are disregarded, the potentials have an almost 'normal' distribution around the mean quantum size of 80 μV with a standard deviation of 20 μV.

Our expectation was that facilitation of the e.j.p.s should increase the number of released quanta without changing their size. Therefore, the average quantum size E_1 of 80 μV and the standard deviation of 20 μV derived from the distribution of e.j.p.s at 1/sec were used for the prediction of a Poisson curve which should fit the e.j.p. size distribution at a stimulation rate of 5/sec. This curve was calculated without any further assumptions. If E_1 and σ_1 are known, the only other needed value is m. This was obtained by dividing the measured mean e.j.p. size ($\bar{E}$) by E_1, giving for m a value of 2·3. The calculated curve fitted very well the observed potential distribution at 5/sec in Fig. 4. We can, therefore, conclude that the quantum size does not change during facilitation, but only the probability for an impulse to release quanta is increased.

The statistical treatment of the results by fitting Poisson curves is somewhat tedious. A simpler relation

$$\log_e \frac{n}{n_0} = \frac{\bar{E}}{E_1} = m \qquad (2)$$

can be derived from the Poisson theorem (1) for $x = 0$. n is the number of stimuli and n_0 the number of failures; this quotient can easily be found. m is again the quantum content, i.e. the average size of the potentials $\bar{E}$ divided by the quantum size E_1. $\bar{E}$ can be measured, therefore formula (2) can be used for calculating the quantum size E_1. This quantum size should be the same as the average size of the spontaneous miniature potentials. And if this formula is used with values obtained at the same synaptic area, but at different frequencies, facilitation should increase m and $\bar{E}$, but the unit size E_1 should remain the same. Table 1 gives the results of this treatment for several of our experiments. As expected, the unit size E_1 is quite constant in spite of considerable changes in quantum content.

It is obvious from Table 1 that the extent to which the single synaptic areas facilitate in the different preparations is quite variable. If this facilitation of the *single* junction ($\bar{E}$) is compared to the average facilitation of all the junctions in a muscle fibre, as represented by the increase of the intracellular e.j.p. (E_i), there can be considerable differences. The increase of the intracellular e.j.p. with a raised stimulus frequency represents only a mean value of the increase in the probability of release of transmitter

in the numerous individual terminals. A striking example of the occasional difference in behaviour of an individual junction, as contrasted to the average seen in the whole muscle fibre, is shown in experiment No. 4. There was no facilitation, but on the contrary, there was a decrease in the number of units released per stimulus (m and $\bar{E}$), i.e. this junction showed 'fatigue'. The sum of the synaptic areas (E_i) of this fibre, however, showed facilitation, as seen in the increase of the intracellular e.j.p.s with increase of frequency. In this experiment the records at the different

TABLE 1

Expt. no.	Stimulation frequency	n	m	$\bar{E}$(μV)	E_0(μV)	E_1(μV)	E_s(μV)	E_i(mV)
1	1/sec	70	0·42	33	—	79	—	0·11
	2/sec	117	0·88	73	72	83	—	0·26
	5/sec	79	2·3	174	—	77	—	0·66
2	1/sec	65	0·44	33	—	76	70	0·08
	2/sec	74	0·94	61	58	65	—	0·16
	5/sec	121	2·2	155	—	70	—	0·40
3	1/sec	115	0·31	15	—	49	48	0·12
	2/sec	236	0·27	12	—	45	—	0·18
	5/sec	211	0·43	18	—	43	—	0·29
4	1/sec	161	1·1	74	—	67	55	0·04
	2/sec	273	1·0	69	—	69	—	0·05
	5/sec	570	0·74	46	45	62	—	0·09
	10/sec	247	0·62	34	—	55	—	0·28
	1/sec	166	0·64	33	—	52	—	0·02

n = number of stimuli; m = average quantum content per stimulus of extracellular e.j.p. determined from $m = \log_e n/n_0$; n_0 = number of transmission failures; $\bar{E}$ = average size of extracellular e.j.p.; E_0 = average size of extracellular e.j.p. preceded by a failure; E_1 = size of quantum determined from $E_1 = \bar{E}/m$; E_s = average size of spontaneous extracellular miniature potentials; E_i = average size of intracellular e.j.p.s.

frequencies were taken in the same sequence as that presented in the table. The response to 1/sec stimulation at the end of the experiment was much smaller than at the start, illustrating the 'fatigue'.

In Table 1 the size of the extracellular spontaneous miniature potentials was used as a control of the calculated unit size. The fact that such good agreement exists is evidence that the spontaneous miniature potentials are not caused by impulse-like activity in the terminals. The extracellular spontaneous potentials in these experiments all fell between e.j.p.s which were set up at rates between 1 and 10/sec. If they had been caused by impulses spontaneously arising in the terminal of the individual junction under the micro-electrode, they would have shown facilitation. Like that of the e.j.p.s, their quantum content should have increased whenever they occurred at higher frequency of stimulation. Since the average amplitude of the spontaneous miniature potentials remains that of the unit quantum, independent of facilitation, their release cannot be caused by a potential change similar to that of the nerve impulse.

Influence of preceding failure

Implied in the statistical treatment of the e.j.p.s recorded from a single junctional area is the assumption that each stimulus reaches this junctional area. The probability for each stimulus to release quanta then is constant in the steady state at a certain frequency. The cases in which no quantum is released, the 'failures', are interpreted as caused by a low probability of release for each stimulus, their number being predicted by the Poisson theorem. Therefore, if a terminal had failed to release a quantum to a certain stimulus, this failure should not affect the probability to release quanta at the next stimulus. Specifically, the facilitation of an e.j.p. should not be affected if it was preceded by a failure. This was tested in experiments in which the effect of decreasing the frequency to one half was very big. The average size (E_0) of the potentials which followed failures was determined in three experiments. The results are contained in Table 1. It can be seen that E_0 was almost identical with the average potential $\bar{E}$ of the whole group. It should be noted that, for instance, in experiment No. 1 the mean potential size dropped to less than half (from 73 to 33 μV) on reducing the stimulation rate from 2/sec to 1/sec. Thus, a great reduction of E_0 would be expected if the stimulus failed to reach the terminal. E_0, however, remained practically unchanged (see Discussion).

Facilitation and spontaneous miniature potentials

Having shown that facilitation consists of an increased probability of quantal release after a nerve impulse, one may suspect that this changed state of the terminal should also express itself in an increased spontaneous release of transmitter quanta (Liley, 1956*a*). An increased frequency of spontaneous miniature potentials has actually been observed under certain restricted conditions. In Fig. 5 a conditioning train of excitatory stimuli at 50/sec for 1 sec was followed by single test stimuli repeated every 2 sec. The test responses were still increased in amplitude after 11 sec. For the same period the average miniature frequency was determined, and only during the first 2 sec was the frequency increased. Such an increase is always seen, provided the conditioning stimulation is not too high in frequency or too long in duration. For instance, if a preparation is stimulated at 1 or 2/sec for several minutes, the spontaneous miniature frequency during this stimulation is always higher than at rest. If, however, the conditioning stimulation is increased, e.g. to 20/sec for 40 sec, as seen in Fig. 6, only a depression of the discharge frequency can be measured for about 4 min. Following the same conditioning stimulation the e.j.p. was facilitated.

From the results it is clear that there is no simple proportionality between

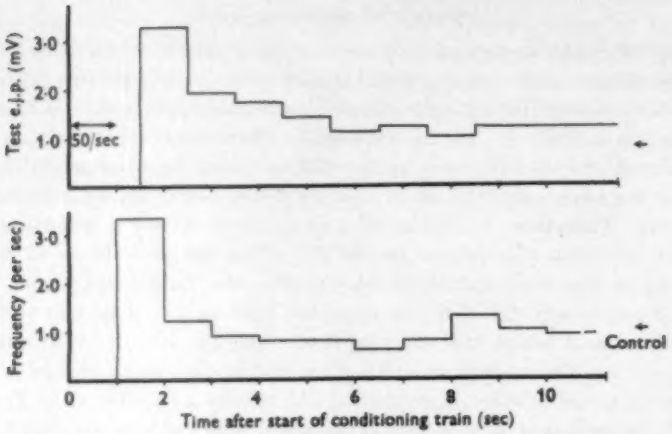

Fig. 5. Post-excitatory facilitation of e.j.p.s and of spontaneous miniature frequency. Excitatory stimulation at rate of 50/sec for conditioning train of 1 sec duration (arrow). Facilitation is tested after train by single e.j.p.s once a second. Upper ordinate: size of test e.j.p.s. Lower ordinate: frequency of spontaneous miniature potentials occurring between test e.j.p.s. Arrows mark control values after several minutes.

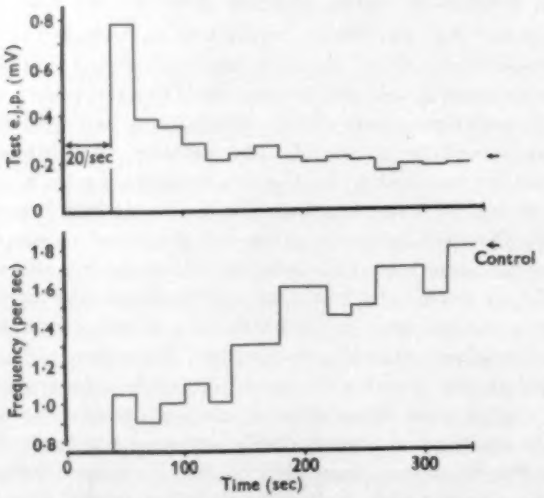

Fig. 6. Post-excitatory effects on e.j.p. size and on miniature potential frequency. Conditioning train of stimuli at 20/sec for 40 sec followed by single test stimuli every 2 sec. Ordinates and abscissa as in Fig. 5.

the extent of facilitation of e.j.p.s and the rate of release of spontaneous miniatures. There is a positive correlation if the conditioning stimulation is at a low frequency. At higher frequencies the spontaneous miniature rates are readily depressed by 'fatigue'. In comparison, the fatigue process affects e.j.p.s only at much higher frequencies. For instance, e.j.p. sizes may decrease after several seconds of stimulation at 100/sec.

DISCUSSION

The main result of this study is the evidence that there occurs during facilitation an increase in the amount of transmitter released from the terminals. It was shown that during facilitation the size of transmitter quanta remained the same, only the probability of release of quanta after a stimulus increased. This finding further strengthens the conclusions of the preceding paper, that the transmission process results from a quantal release of transmitter. Our results show that the site of facilitation is in the nerve terminals.

There are two other mechanisms which are frequently assumed to play a role in bringing about facilitation. The first is an increased recruitment of previously blocked nerve terminals. In the facilitation which we have studied this mechanism played no significant role for the following reasons. First, at low frequencies, when the junction shows relatively little facilitation, one sees no more failures than are predicted by Poisson's theorem. Secondly, an e.j.p. after a failure shows just as much facilitation as when no failure had occurred. If the failure had been a conduction block, the effective stimulation frequency would have been reduced, resulting in less facilitation and smaller e.j.p.s.

When searching for junctions, we explored the muscle surface while stimulating continuously at 1/sec. Therefore, the active spots we found may represent a selected group of terminals which release quanta already at a low frequency. If terminals exist which are blocked at such low frequencies, but are unblocked at high frequencies, we may not detect them. Such an assumption appears unnecessary, because at frequencies between 1 and 10/sec the range of facilitation at single junctional areas was quite adequate to account for the facilitation of the e.j.p. in the whole fibre as recorded intracellularly.

A second mechanism of facilitation which is frequently discussed is an increase in post-synaptic sensitivity. This again appears to be excluded by the present experiments. A post-synaptic mechanism implies that the increased e.j.p.s during facilitation are brought about by a bigger depolarization caused by the same amount of transmitter. However, (1) the unit size did not change during facilitation, and (2) the size of the spontaneous miniature potentials remained the same (cf. also Hutter,

1952, del Castillo & Katz, 1954*a*). If there was post-synaptic facilitation both these values should increase.

How the increase in the probability of release in the terminals is brought about is unknown (cf. Curtis & Eccles, 1960, for a recent discussion). There is good evidence that the release itself is in some way dependent on the membrane potential change (del Castillo & Katz, 1954*b*; Liley, 1956*b*; Hagiwara & Tasaki, 1958). Therefore, one may speculate that the increased probability of release is due to some residual long-lasting after-potential which then affects the size of the subsequent impulse and thereby makes it more effective (Larrabee & Bronk, 1947; Lloyd, 1949; del Castillo & Katz, 1954*b*; Koketsu, 1956; Eccles & Krnjević, 1959; Wall & Johnson, 1958). We do not know in any detail, however, what the normal impulse activity of terminals is. It is, for instance, quite speculative whether the conducted impulse which is recorded in the axon actually propagates to the site of transmitter release. It may be that only the electrotonic spread of the action potential in the axon reaches release sites. It may then be the conditions of passive current spread which are improved during consecutive stimulation. However, there are other possibilities and one actually need not postulate a mechanism which is reflected in the membrane potential. The chemical link between potential and the transmitter packets is just as likely to be involved (cf. Dudel & Kuffler, 1961*b*).

Our results on the progressive decrease of quantal release from junctions indicate that some of the 'fatigue' of neuromuscular transmission can be accounted for by decreased transmitter output by the terminals. Of course, at high frequencies of stimulation progressive conduction block in the nerve has been observed in some of our preparations. This is a different type of 'fatigue' and is similar to that studied in the rat neuromuscular junction by Krnjević & Miledi (1958).

Again, with the facilitation mechanism, as with the quantal mechanism of activation in general, the close analogy with vertebrate junctions should be pointed out (del Castillo & Katz, 1954*a*; Liley, 1956*a*; Boyd & Martin, 1956). This makes it likely that we are dealing with a rather general property of synaptic transmitter release mechanism.

SUMMARY

1. In the abductor of the dactyl in the crayfish the excitatory junctional potentials (e.j.p.s) increase in amplitude with an increased frequency of motor nerve stimulation. This process of facilitation has been analysed by recording with extracellular micro-electrodes from only one of the finely localized individual junctional areas which are widely distributed along the muscle surface. Simultaneously, an intracellular electrode registered the integrated junctional activity which takes place in the entire muscle fibre.

2. During low-frequency stimulation when the intracellularly recorded e.j.p.s are small, motor nerve impulses release excitatory transmitter only intermittently at individual junctional areas. Statistical treatment has shown that the transmitter is released in quanta and that during facilitation the number of released quanta increases, while the quantum size remains unchanged. Thus, facilitation can be described formally as an increase in the probability of release of quanta by a nerve impulse.

3. Facilitation is not brought about by invasion of nerve terminals which previously have not been reached by the nerve impulses. Nor does an increased sensitivity of the post-synaptic membrane to the transmitter contribute appreciably to facilitation.

4. Some observations indicate that 'fatigue' at junctions is due to progressively diminished probability of release of transmitter quanta.

5. Following a train of conditioning stimulation single e.j.p.s may be facilitated for a minute or longer. After such a conditioning train, there usually occurs a brief (several seconds) increased frequency of spontaneous miniature potentials, followed by a long period (up to 4 min) of decreased spontaneous discharge rate.

6. The mechanism of neuromuscular facilitation in the crayfish resembles closely that observed in vertebrate junctions.

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PRESYNAPTIC INHIBITION AT THE CRAYFISH NEUROMUSCULAR JUNCTION

BY J. DUDEL* AND S. W. KUFFLER

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There are three possible ways in which synaptic inhibition may reduce excitation. (1) Inhibition may reduce the amount of excitatory transmitter that is released from nerve terminals when they are activated by a nerve impulse; this is presynaptic inhibition. (2) The reaction of the post-synaptic receptors to a constant amount of released transmitter may be reduced, for instance by competition. (3) The post-synaptic membrane may be altered in such a way that the depolarizing action of the excitatory transmitter is opposed.

There exists good evidence for the last alternative only, namely, that the membrane conductance of the post-synaptic region is increased, with the membrane potential staying near the resting potential. This inhibitory mechanism of a specific post-synaptic permeability increase (to K^+ and/or Cl^-) seems to be widespread in different species and has been demonstrated in the vertebrate heart, the mammalian central nervous system, in crustacea at neuromuscular junctions and nerve cell synapses, as well as in various other preparations (Fatt & Katz, 1953; Coombs, Eccles & Fatt, 1955; Kuffler & Eyzaguirre, 1955; Trautwein & Dudel, 1958; for a recent review see Kuffler, 1960).

In this study on the crayfish neuromuscular junction we present evidence for a presynaptic mechanism. The inhibitory nerve impulse acts on the excitatory nerve terminals and decreases the probability of release of quanta of excitatory transmitter. A preliminary report has appeared (Dudel & Kuffler, 1960).

METHODS

As previously described, the abductor muscle of the dactylopodite in the crayfish was used (Dudel & Kuffler, 1961*a*). For altering the muscle membrane potential the current-passing electrode was filled with saturated potassium citrate solution (Boistel & Fatt, 1958). These electrodes were preferable because they carried more readily maintained currents of up to 10^{-8} A.

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RESULTS

Conductance increase during inhibition

Some features of the conductance-increase mechanism, first demonstrated by Fatt & Katz (1953), are presented here largely to make clear the distinction from presynaptic inhibition. Inhibitory stimulation reduces the potential change that is produced when a current is applied across the junctional membrane, i.e. the resistance of the membrane has decreased

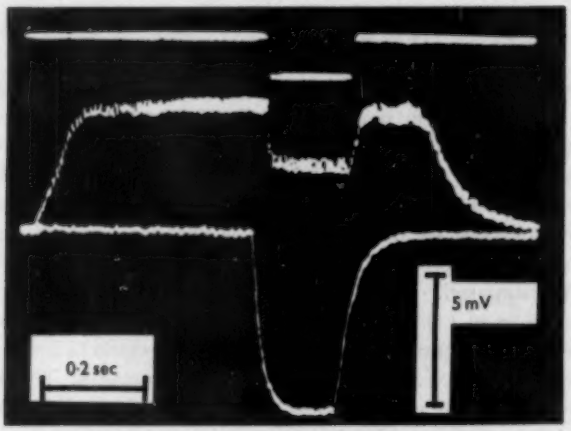

Fig. 1. Conductance increase during depolarizing inhibitory junctional potential in abductor of the dactyl. One recording and one current-passing electrode inserted into a muscle fibre. Current pulses of $1{\cdot}5 \times 10^{-9}$ A monitored in upper sweep were passed across the membrane at rest (lower sweep) and during inhibitory potential. Stimulation frequency of inhibitory nerve was 150/sec. Note reduction of the electrotonic potential from 7 to 2 mV by inhibition.

during inhibition. This is seen in Fig. 1, where two sweeps are superimposed. The lower record gives the electrotonic potential in the resting state of the membrane caused by the current pulse (upper trace). In the middle trace the inhibitory axon was stimulated by a short train of inhibitory impulses at 150/sec and a small depolarization of the fibre resulted. The same current pulse, if applied during the plateau of inhibitory junctional potentials (i.j.p.s), set up a much reduced electrotonic potential with a more rapid time course. The calculated membrane conductance during inhibition, as measured by this method, was frequently increased ten times above the resting value.

The most characteristic feature of the conductance type mechanism is the reversal of the i.j.p. near the resting potential. The method of determining the reversal potential was used routinely in the present studies.

An example is shown in Fig. 2. The membrane was depolarized by passing current through a second intracellular micro-electrode. Starting with a resting potential of 80 mV the i.j.p.s became negligibly small at 72 mV and reversed their direction with additional depolarization. Arrows mark the reversal potential.

The reversal potential obtained in Fig. 2 may not indicate exactly the true inhibitory equilibrium potential, being slightly too positive, because the current electrode does not depolarize the whole fibre uniformly (see Burke & Ginsborg, 1956, for discussion of an analogous problem). Here, however, this introduces only a small error. The reversal or equilibrium

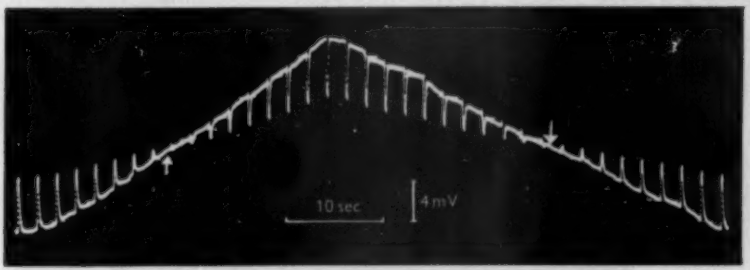

Fig. 2. Trains of inhibitory junctional potentials (150/sec for 0·2 sec) repeated every 2 sec. Resting potential at start 80 mV shifted to 61 mV by passing current through a second intracellular micro-electrode. Arrows mark the reversal potential of inhibitory potentials at 72 mV.

potential for i.j.p.s was also determined by depolarizing the fibre uniformly by excess K^+, instead of by current, and no significant difference from the result of Fig. 2 (arrows) was noted.

An additional mechanism for inhibition

In their original work Fatt & Katz (1953) thought that the reduction of excitatory junctional potentials (e.j.p.s) was too great to be brought about by a conductance increase alone, and they suspected an additional mechanism (cf. Kuffler & Katz, 1946). Experimental conditions have now been found in which the post-synaptic conductance increase mechanism cannot account for any of the observed reduction of the e.j.p. In Fig. 3 single excitatory stimuli set up excitatory junctional potentials of 2 mV (Fig. 3*A*) Single inhibitory stimuli caused a small depolarization of about 0·2 mV (Fig. 3*C*). The inhibitory equilibrium level, however, as found by the method shown in Fig. 2, and further determined by K depolarization (above), was at the dotted line 6 mV more positive than the resting potential. In this situation in which the *excitatory* junctional potential does not reach the *inhibitory* reversal level, the inhibitory conductance

mechanism cannot reduce the e.j.p. If the i.j.p. is timed to fall on the peak of the e.j.p., it should still depolarize, since the i.j.p. is still 'below' (more negative than) its reversal potential. In fact, it should add to the e.j.p. This seemed to be the case in Fig. 3*B*, where the combined potential, if changed at all, was somewhat bigger. To our surprise, however, if the i.j.p. was timed to precede the e.j.p. by 3 msec, the e.j.p. was greatly reduced (Fig. 3*D*). This cannot be explained by a mechanism of inhibitory conductance increase, which can shift the membrane potential only toward the inhibitory reversal level. Therefore, an additional mechanism had to be postulated. Still further evidence for an additional mechanism will be presented below with the results on extracellular recording (Figs. 6–8).

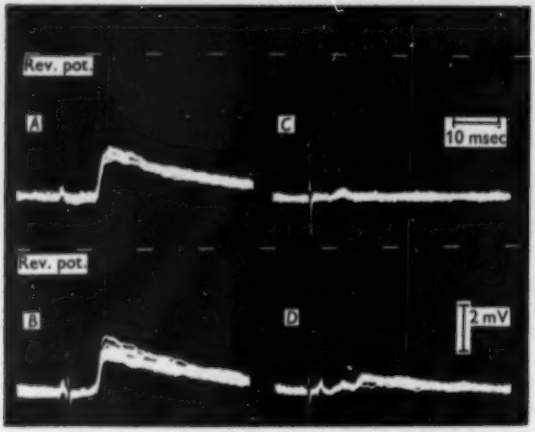

Fig. 3. Intracellular records from abductor muscle fibre. Resting potential 86 mV, inhibitory reversal level (rev. pot.) 6 mV more positive (interrupted line), K^+ in solution reduced to 2·7 mM. *A*: single excitatory junctional potential (e.j.p.) of 2 mV. *C*: single inhibitory junctional potential (i.j.p.) depolarizing 0·2 mV. *B*: inhibitory axon stimulated 1·5 msec after excitatory axon; i.j.p. coincides with peak of e.j.p. *D*: inhibitory axon stimulated 3 msec before excitatory axon; i.j.p. seen before e.j.p. which is greatly reduced.

A close examination of Fig. 3*D* reveals that the rising phase of the small i.j.p. was over when the e.j.p. started. The maximum of the inhibitory conductance increase occurs during the rise of the i.j.p., and the residual conductance increase during the falling phase of the i.j.p. must have been relatively small at a time when the reduction of the subsequent e.j.p. was seen to be maximal.

To obtain the above type of reduction of e.j.p.s the timing of inhibitory stimuli in relation to the excitatory ones was quite critical. If the inhibitory stimulus preceded the excitatory one by 1–3 msec the effect was

maximal and then declined over a further 3–5 msec. The importance of timing of the excitatory–inhibitory stimuli for reducing excitatory potentials, as is shown in Fig. 4, has been known for a long time and similar curves were obtained by Marmont & Wiersma (1938), Kuffler & Katz (1946) and Fatt & Katz (1953). The significance of the timing effect for our experiments is that it revealed a type of inhibition that could not be accounted for by the conductance-increase mechanism.

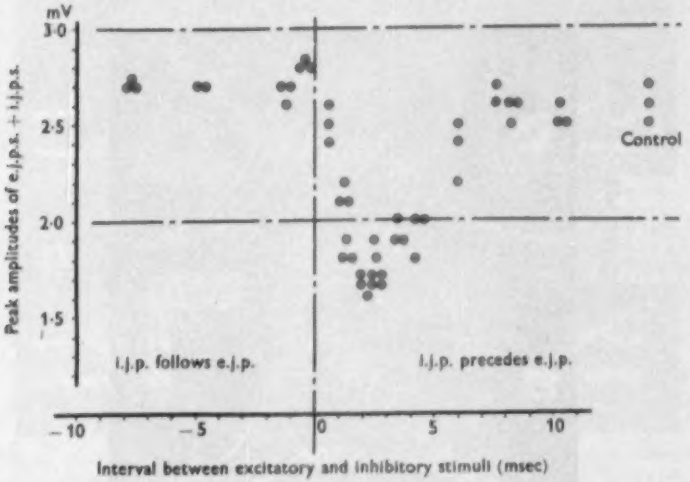

Fig. 4. Experiment as in Fig. 3. The inhibitory equilibrium potential was 6 mV more positive than resting potential. Control gives size of e.j.p. without any inhibitory stimulation.

Another way of showing the influence of timing of impulses is illustrated in Fig. 5. Inhibitory and excitatory nerves were stimulated at rates of 100/sec for 120 msec. In Fig. 5*B* each inhibitory nerve stimulus was delivered 2·5 msec before the excitatory one, while in Fig. 5*A* it was given 8·0 msec before each excitatory stimulus. The e.j.p.s were much more reduced in Fig. 5*B*, where the timing was maximally effective.

Effect of inhibition on extracellularly recorded e.j.p.s

The existence of a second type of inhibition in addition to the post-synaptic conductance-increase mechanism can also be shown by recording extracellularly from individual junctional areas with micro-electrodes. This recording method has the advantage that the quantum content of the synaptic potentials can be determined statistically (Dudel & Kuffler,

1961*a*, *b*), and more specific results on the nature of the second type of inhibition can be obtained.

In the lower sweep of Fig. 6*A* are shown extracellular records from a single junctional area. The e.j.p.s are negative, in contrast to the intracellular junctional potentials which are recorded simultaneously (upper sweep). The latter are made up of activity in the entire muscle fibre. If properly timed inhibitory impulses were added in Fig. 6*B*, the intracellular e.j.p. heights were reduced, as one would expect. The significant change in

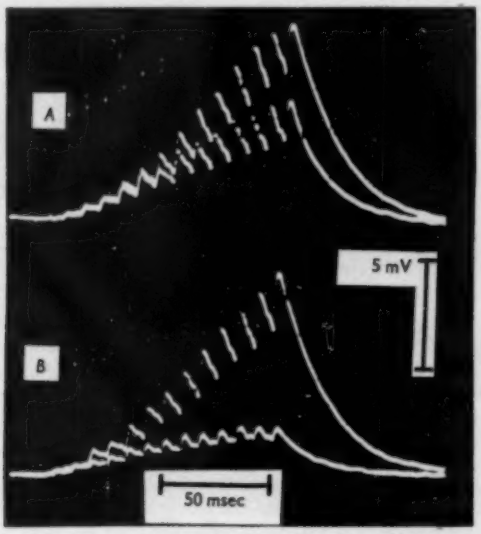

Fig. 5. Minimally and maximally effective timing for inhibition. Intracellular recording, resting potential near 75 mV. *A*: upper sweep, excitatory stimuli at 100/sec for 120 msec; lower sweep, inhibitory stimuli precede excitatory stimuli by 8 msec. *B*: same excitatory and inhibitory trains as in *A*, but in lower sweep the inhibitory stimulus precedes each excitatory one by 2·5 msec, resulting in maximally effective reduction of the e.j.p.s.

the extracellular records from the single junction was the increased number of failures (arrows), i.e. the excitatory impulse failed to release packets of transmitter. At the same time, the mean amplitude of the 'hits' was reduced in size (see below, Table 1).

In Fig. 7 more detail is included in a fast sweep. Three consecutive sweeps were superimposed, repeated at a rate of 5/sec. Fig. 7*A* shows excitation alone, with the stimulus artifacts in the lower record followed by the motor nerve impulse (arrow) arriving near the junction. Then two negative extracellular e.j.p.s appeared on the lower sweep, which lasted a

little longer than the rising phase of the intracellular e.j.p.s in the upper sweep. There was, in this sequence of three stimuli, one failure of transmission at the single junctional area. The intracellular e.j.p.s did not fluctuate greatly. In Fig. 7*B* an inhibitory stimulus (first artifact)

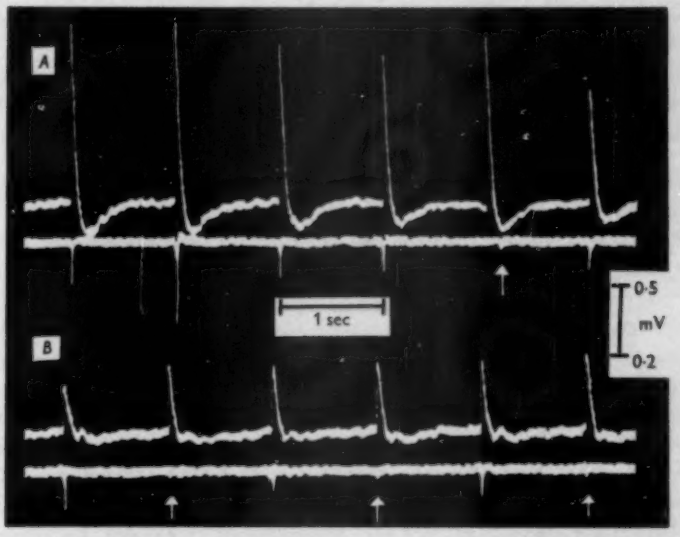

Fig. 6. Intracellular records (upper sweeps) from a muscle fibre and simultaneous extracellular measurements from single junctional area (lower sweep) at stimulation rate of 1/sec. *A*: excitatory stimulation alone. *B*: an inhibitory stimulus is added, preceding the excitatory one by 2 msec; e.j.p.s are reduced and number of transmission failures in single junctional area (arrows) increased. The diphasic portion in the intracellular records is due to AC amplifier.

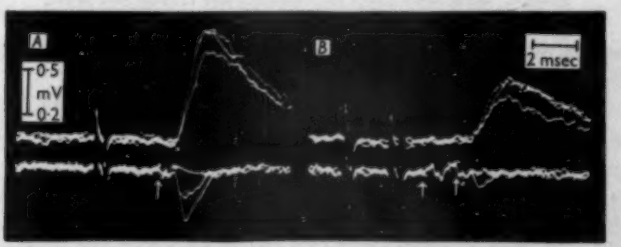

Fig. 7. Simultaneous intra- and extracellular records, as in Fig. 6, three consecutive junctional potentials superimposed at rate of 1/sec. *A*: e.j.p.s alone; failure of transmission occurs once in extracellular records; arrow marks extracellularly recorded motor nerve impulse near the junction. *B*: inhibitory stimulus preceding excitatory one by 2 msec, reducing the intracellular e.j.p.'s and resulting in two failures of transmission at the single junctional area; arrows mark nerve impulses.

preceded the excitatory one by 2 msec. On the lower beam we now see two extracellular nerve impulses (arrows), the first being inhibitory. They are 1·5 msec apart, a little less than the stimulus interval, presumably owing to different conduction distances and speeds of propagation. Again, all the intracellular e.j.p.s were reduced and in the lower beam the number of transmission failures was increased from 1 to 2. Only one e.j.p. of about 50 μV remains (see below).

Figures 6 and 7 show that inhibition can considerably reduce the average extracellular response. This reduction of the extracellular e.j.p. cannot be brought about by an increased membrance conductance. This

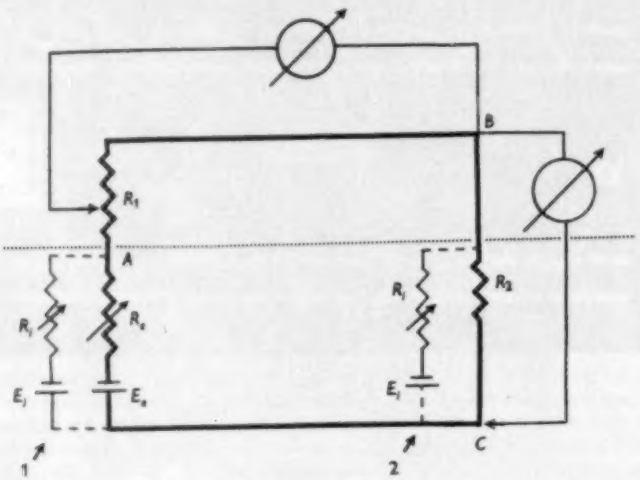

Fig. 8. 'Equivalent circuit' diagram showing external and internal recording conditions. Long dotted line indicates surface of fibre (see text).

point can be readily appreciated if the extracellular recording conditions are analysed.

Figure 8 gives an 'equivalent circuit', modified from that given by del Castillo & Katz (1956, Fig. 17*b*) for recording extracellularly from vertebrate end-plates. A is the active excitatory junctional area, R_e is the membrane resistance of this area and E_e is the driving force for the e.j.p.: R_1 is the resistance of the small extracellular fluid volume outside the junction, where the synaptic current density is very high. B represents the distant indifferent electrode. R_2 is the 'input resistance' (i.e. between a point inside the fibre (C) and the extracellular fluid (B)). The intracellular electrode records between C and B, the extracellular electrode between B and a part of R_1. There are two alternative locations for an inhibitory

membrane conductance increase to act on this circuit. First, the inhibitory synaptic membrane could be so close to the excitatory one that R_e and R_i are in parallel and the inhibitory and excitatory synaptic currents flow through the same restricted extracellular fluid volume with a resistance R_1. The second alternative is for the inhibitory synaptic area to be some distance away from A, so that it is in parallel with R_2. It can be shown that in both cases the reduction of R_i (inhibitory conductance increase) does not diminish the extracellularly recorded e.j.p. If in alternative (1) an appreciable current were produced, extracellular potentials should be recorded on inhibitory stimulation. We have not seen such potentials. In alternative (2) the inhibitory resistance decrease works essentially as a shunt to R_2. R_2 is small compared with $R_1 + R_e$, as is seen from the fact that the intracellular junctional potential is much smaller than its driving force E_e, as pointed out by del Castillo & Katz (1956). Thus shunting R_2 cannot diminish current flow through R_1; it could only slightly increase it, producing a larger extracellular e.j.p.

Therefore, the reduction of the extracellularly recorded e.j.p.s during optimally timed inhibition provides another demonstration of an inhibitory mechanism which cannot be accounted for by an increase in membrane conductance. By themselves the above results do not allow us to distinguish the site of action of this other inhibitory mechanism. The site may be presynaptic, with less transmitter released per impulse, or it may be post-synaptic, for instance, with competition taking place between excitatory and inhibitory transmitters. By the use of a statistical analysis, however, it was possible to show that less transmitter was released during inhibition and that the site of action must therefore be presynaptic. That is, presynaptic inhibition should reduce the number rather than the size of excitatory transmitter quanta. Competitive inhibition would have the converse effect. Information about quantum size and number can be obtained by statistical treatment of our extracellular records from single synaptic areas.

Statistical treatment

Figure 9 gives the size distribution of extracellular e.j.p.s from a single junctional area with and without inhibition. Each graph contains 1000 counts. At a frequency of 5/sec there are relatively few transmission failures in the uninhibited junction. With inhibition the failures increase from 72 to 570 and there are fewer large e.j.p.s. For instance, in the uninhibited junction there are 32 e.j.p.s of 150 μV, while inhibition reduces them to 2. The whole distribution is shifted to the left.

The inhibited e.j.p.s were sharply grouped around a peak of about 40 μV. They must have a very low quantum content, because more than half the responses were failures. A fitting Poisson curve could, therefore,

be determined readily for these potentials by the method previously used (Dudel & Kuffler, 1961*a*). The theoretical curve (interrupted line) fits the histogram well. The unit size derived from this Poisson distribution was $E_1 = 40\ \mu V$ and the number of quanta released by one stimulus was $m = 0{\cdot}56$.

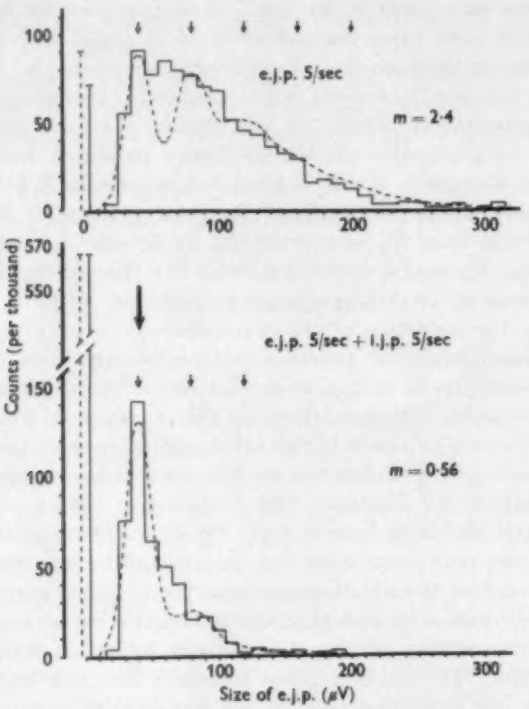

Fig. 9. Histograms giving size distribution of extracellularly recorded e.j.p.s from a single junctional area at stimulation rate of 5/sec. Upper graph, junction not inhibited. Lower graph, inhibitory impulses precede excitatory ones by 2 msec; note increased number of failures from 72 to 570. Interrupted line is calculated theoretical (Poisson) distribution for unit quantum size E_1 (40 μV) with standard deviation σ_1 (10 μV), and an average quantum content per stimulus m. Small arrows indicate multiples of quantum size, heavy arrow gives average size of spontaneous miniature potentials.

The question now was whether the uninhibited e.j.p.s had the same quantum size as the inhibited ones. This could be tested by calculating a Poisson curve for the uninhibited e.j.p.'s, using only the 40 μV unit size obtained above, and the average size of the uninhibited e.j.p.s (see Dudel & Kuffler, 1961*b*). The calculated curve was a reasonably good fit.

Thus it was shown that inhibition of the e.j.p.s could be described as a reduction of the average quantum content from 2·4 to 0·6 while the size of the unit remained unchanged.

An independent control of the size of the quantum E_1 is derived from the average size of the spontaneous miniature potentials. Fourteen spontaneous potentials were observed during the experiment of Fig. 9, with an average size of $E_s = 41\ \mu V$ and a standard deviation of $\sigma_1 = 11\ \mu V$. E_s thus agrees well with the quantum size E_1.

The possibility could be considered that the inhibitory effect on the release of excitatory transmitter fluctuates over a wide range, possibly in a quantal way. This would result in a large fluctuation of the probability for the release of quanta of excitatory transmitter. However, the Poisson theorem assumes that the probability of release of quanta is the same for each stimulus. A wide fluctuation of this probability would distort the Poisson distribution considerably. Since we were able to fit the inhibited e.j.p.s by a Poisson distribution, the inhibition of the excitatory transmitter release should be relatively constant with each inhibitory stimulus. If the inhibitory transmitter is released in quanta, the quantum content of the inhibitory potentials would have to be relatively high even at low frequencies. Such an assumption would be in line with the observation that at low stimulation frequencies (around 1/sec) the amplitude of the intracellular i.j.p.s fluctuated less than that of the intracellular e.j.p.s.

A simpler statistical treatment consists in using a relation derived from Poisson's theorem. It relates the number of trials (n) and failures (n_0) of transmission n/n_0 to mean quantum content m. m is given by the average size $\bar{E}$ of e.j.p.s divided by the unit size E_1, as treated in the previous papers (Dudel & Kuffler, 1961*a*, *b*).

$$\log_e \frac{n}{n_0} = \frac{\bar{E}}{E_1} = m. \qquad (1)$$

All values except E_1 can be determined directly and so E_1 can be calculated.

Table 1 gives a comparison of e.j.p.s with and without inhibition in six experiments. The calculated values for E_1 (unit size) agree well for inhibited and uninhibited junctions. In the same experiments the average size of spontaneous potentials (E_s) was measured and, as expected, was found to be in close agreement with E_1 (Dudel & Kuffler, 1961*a*). The last column (E_i) gives the intracellular e.j.p.s, i.e. the integrated activity from the whole fibre. A comparison of the intracellular e.j.p. reduction during inhibition with the reduction of extracellular e.j.p.s gives an interesting picture of the variability of activity in individual junctions. The differences in reduction can be quite striking. In experiment No. 3 the average extracellular e.j.p. size is radically reduced to less than 1/20 by inhibition, while the intracellular e.j.p. is only halved. This must mean that the effectiveness of inhibition at individual junctional areas varies greatly.

In the preceding study (Dudel & Kuffler, 1961*b*) it was shown that failure by a nerve impulse to set up an e.j.p. at a single junctional area did

not alter the average size of the next e.j.p. and did not affect its facilitation. This was in agreement with the basic assumption made for the Poisson treatment, that each stimulus has the same probability of releasing quanta, i.e. each stimulus reaches the junction. The rather noncommittal term 'stimulus' is used here because we do not know whether the impulse seen in the axon actually travels right to the terminals. It was found that also in groups of extracellular e.j.p.s which were inhibited by optimally timed inhibition, failures of transmission did not affect the size of succeeding

TABLE 1

Expt. no.	Stimulation frequency	n	m	$\bar{E}$ (μV)	E_1 (μV)	E_s (μV)	E_i (mV)
1	5/sec	961	1·3	85	65	71	1·47
	5/sec + inhib.	1375	0·72	50	69	—	1·17
2	5/sec	728	2·8	94	34	41	1·2
	5/sec + inhib.	699	0·57	24	42	—	0·45
3	5/sec	228	1·3	35	30	30	1·7
	5/sec + inhib.	308	0·03	1·2	40	—	0·8
4	2/sec	200	0·60	30	49	51	0·12
	2/sec + inhib.	136	0·28	14	48	—	0·08
5	5/sec	264	0·79	34	43	—	0·25
	5/sec + inhib.	167	0·31	13	42	—	0·11
6	5/sec	570	0·74	46	62	55	0·09
	5/sec + inhib.	409	0·50	31	62	—	0·07

n = number of stimuli; m = average quantum content of extracellular e.j.p. determined from $m = \log_e n/n_0$; n_0 = number of transmission failures; $\bar{E}$ = average size of extracellular e.j.p.; E_1 = size of quantum determined from $E_1 = \bar{E}/m$; E_s = average size of spontaneous extracellular potential; E_i = average size of intracellular e.j.p.

e.j.p.s. The average size of the e.j.p.s preceded by a failure was the same as the average size of the whole group. This finding further justifies the treatment of inhibited e.j.p.s by the Poisson analysis (see Discussion).

The statistical treatment has shown that inhibition can be described as a reduction of the number of released quanta, while the quanta themselves are not changed. Thus, optimally timed inhibitory stimuli act presynaptically on the excitatory system.

The effect of inhibition on spontaneous miniature potentials

Having shown that appropriately timed inhibitory impulses act presynaptically, their effects on the post-tetanic increase of the frequency of spontaneous miniature potentials (Dudel & Kuffler, 1961*b*) are of interest. The experiment was done in the following way: Trains of excitatory stimuli, for instance, at 75/sec for 200 msec each, were repeated every 2 sec. The miniature frequency was measured in the intervals between the trains and was found increased above the rate seen in the non-stimulated muscle. The excitatory trains were then repeated with optimally (as

Fig. 5*B*) and minimally (Fig. 5*A*) effective inhibitory timing. A count of spontaneous miniature potentials between the trains showed that they were reduced when the excitatory–inhibitory timing was set for maximal reduction of the e.j.p.s. A tabulation of the results from two experiments is shown in Table 2. The decrease of miniature frequency was significant at the 99·9 % level. Since miniature potentials are released presynaptically, anything that changes the frequency of release presumably acts presynaptically. This effect therefore shows once more that inhibition has a presynaptic effect.

TABLE 2

Expt. no.	Conditioning train	Inhibition timing	Measurements *n*	Miniature frequency	S.E.
1	50/sec	min.	106	1·44/sec	0·06
	50/sec	optimal	159	1·13/sec	0·06
2	75/sec	min.	148	2·61/sec	0·09
	75/sec	optimal	94	1·98/sec	0·10

One more observation, in which inhibition is likely to affect the presynaptic terminals, concerns post-tetanic facilitation. A train of conditioning excitatory stimuli, for instance, at 50/sec for 1 sec, was followed by a facilitation of single test stimuli for 10 sec or longer. If in the conditioning train inhibitory stimuli were set to precede each excitatory one by 2 msec (as in Fig. 5*B*), the subsequent post-tetanic facilitation was significantly reduced. If, however, the same inhibitory impulses were timed to arrive at the junction after the excitatory ones, there was no noticeable effect on post-tetanic facilitation.

Relationship of inhibition to facilitation

In a formal sense the reduction of the probability of release by inhibition is the opposite of facilitation. Thus presynaptic inhibition could be interpreted as a removal or prevention of facilitation. If this were true, presynaptic inhibition would reduce e.j.p.s to their non-facilitated size. As a consequence, greatly facilitated, large e.j.p.s would be reduced by a greater factor than the smaller, less facilitated ones. This, however, is not so, because it was found that if e.j.p.s were set up at 10 or 20/sec they were reduced by optimally timed presynaptic inhibition by the same proportion as the much less facilitated e.j.p.s at 1/sec. An illustration is given in Table 3. The percentage of e.j.p. reduction seen in the last column clearly does not depend on the frequency of excitatory stimulation. This reduction by the same proportion, independent of e.j.p. size, seems remarkable (see Discussion). To obtain it, however, one has to take the precaution in these experiments of keeping the inhibitory facilitation constant. For instance,

the inhibitory frequency was left unchanged at 10/sec in the above tests. The change in excitatory stimulation rate from 10/sec to 1/sec was simply made by cutting out nine of the ten stimuli by an electronic gate (decade counter). Only the inhibitory i.j.p. which precedes the e.j.p. by 2 msec will have a presynaptic inhibitory action. The others precede the e.j.p. by more than 100 msec and have no inhibitory effect (cf. Fig. 4); they only serve to keep facilitation of the i.j.p.s constant.

The above experiments had a very different outcome if both excitatory and inhibitory frequencies were reduced together. Then there was much less reduction of the e.j.p. at a lower frequency. An example is given in experiment No. 3, Table 3. With inhibitory stimulation at 2/sec there was

TABLE 3

Expt. no.	e.j.p. frequency	e.j.p. size (mV)	i.j.p. frequency	e.j.p.+i.j.p. size (mV)	Reduction of e.j.p. (%)
1	20/sec	16·0	20/sec	7·0	56
	2/sec	1·6	20/sec	0·7	56
	10/sec	4·5	10/sec	2·4	47
	1/sec	0·7	10/sec	0·37	47
2	5/sec	8·5	5/sec	5·8	32
	0·5/sec	3·6	5/sec	2·8	22
	10/sec	11·0	10/sec	7·0	36
	1/sec	4·4	10/sec	2·8	36
3	2/sec	2·0	2/sec	1·9	5
	20/sec	15·2	20/sec	10·2	33
	2/sec	2·4	20/sec	1·6	33

practically no reduction of e.j.p.s (5 %). This experiment demonstrates that presynaptic inhibition also is facilitated like the post-synaptic inhibitory potential (Fig. 1*B*, Dudel & Kuffler, 1961*b*).

The effect of gamma-aminobutyric acid (GABA) and presynaptic inhibition

In search of a mechanism for presynaptic inhibition one has to examine whether the inhibitory nerve acts on the excitatory terminals electrically, i.e. by current spread, or chemically, by the action of a transmitter. Our only available evidence indicates that there are chemoreceptor sites on the excitatory fibre endings. We were able to mimic presynaptic inhibition by GABA.

It is known that GABA increases the post-synaptic membrane conductance in a manner similar to inhibitory nerve stimulation. Accordingly it shifts the membrane potential to the inhibitory equilibrium level, i.e. the potential change due to GABA reverses at the same potential at which inhibitory potentials reverse (Kuffler, 1960). For the present test one needs conditions in which the known inhibitory post-synaptic conductance increase by GABA can be excluded, as it was excluded for neural inhibi-

tion in the experiment of Fig. 3. These requirements were met in the following manner. In the preparation of Fig. 10 the inhibitory reversal potential (rev. pot.) was 7 mV more positive than the resting potential (interrupted line), determined as in Fig. 2. Excitatory stimuli at 10/sec set up e.j.p.s, which after a period of facilitation reached a fairly steady height near 5·5 mV. If, for instance, 5×10^{-5} M or a stronger concentration of GABA was applied (not illustrated), the resting potential became depolarized toward the inhibitory reversal potential (cf. Kuffler, 1960, Fig. 11) and the e.j.p.s further added to the depolarization, their peaks exceeding the reversal potential. At certain concentrations of GABA

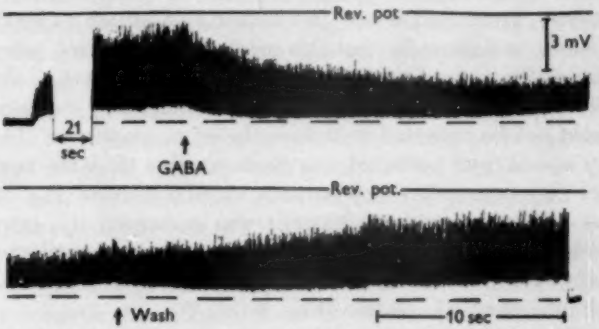

Fig. 10. Second 'inhibitory' action of GABA. Intracellular records of e.j.p.s at stimulation rate of 10/sec. Peaks of e.j.p.s do not reach the inhibitory reversal potential (rev. pot.), which was 7 mV more positive than the resting potential (interrupted line). 10^{-5} M GABA reduced e.j.p.s moving their peaks away from the reversal potential. The reversal potential for GABA coincided with the inhibitory reversal potential.

(10^{-5} M in the preparation of Fig. 10) no depolarization of the membrane was noted while the e.j.p. peaks were reduced, *away* from the inhibitory equilibrium potential. (Note a small hyperpolarization, due to the non-specific 'wash effect' of the applied solution.)

In interpreting the result of Fig. 10 one needs to point out that the known conductance increase of the post-synaptic membrane by GABA can shift the potential only toward the GABA equilibrium potential. As long as the e.j.p. peaks are *below* the inhibitory equilibrium level, their diminution away from this potential cannot be accounted for by an inhibitory conductance increase. Therefore, GABA has, in addition to its post-synaptic conductance increase, a second inhibitory action on the e.j.p. On the basis of the analogy with presynaptic inhibition described in this study, the effect of GABA is probably due to its action on the motor endings.

DISCUSSION

Our results concerning the conductance increase in crustacean muscle fibres during inhibition are in full agreement with those of other workers (Fatt & Katz, 1953; Boistel & Fatt, 1958; Grundfest, Reuben & Rickles, 1959; Hagiwara, Kusano & Saito, 1960). The conductance increase in crayfish muscle during inhibition is predominantly an increase in the permeability to Cl^-, a conclusion confirmed once more by our finding that changing the membrane potential by excess K^+ did not significantly alter the inhibitory equilibrium potential (Fig. 2).

An examination of the evidence for presynaptic inhibition may be made in two stages. First, that a new mechanism in addition to conductance increase exists, and secondly that this mechanism is, in fact, presynaptic. The evidence for an additional mechanism is quite direct. Two experimental situations were presented in which an inhibitory conductance increase could not be expected to reduce the e.j.p. amplitude. In one the inhibitory equilibrium potential was more positive than the peak of the e.j.p.s, yet they were reduced by properly timed inhibition (Fig. 3). In the other the excitatory synaptic current was measured by extracellular micro-electrodes. This synaptic current was not changed by the inhibitory post-synaptic conductance increase. We found, however, very strong reductions with inhibitory stimulation (Figs. 6 and 7).

The positive evidence for the presynaptic mechanism is less direct than the evidence which excludes the post-synaptic conductance increase in specific instances. The principal new result reported here is that during inhibition the size of the released quanta was unchanged, while their number was reduced, i.e. less transmitter was released. This alone requires that the process be presynaptic provided the quanta are released presynaptically. The reasons for the latter assumption were presented in the first two papers in this series (Dudel & Kuffler, 1961*a*, *b*). It should be recalled that these papers did not contain direct evidence for the presynaptic release of quanta but showed the close and detailed analogy with the quantal release in the vertebrate junction; there the evidence for quantal release from the presynaptic terminals was complete (Katz, 1958).

There are numerous questions left about the details of the presynaptic events. Not even the structural relationship between the excitatory and inhibitory terminals is known. Under the light microscope the axons run together and split up together into fine arborizations on the muscle surface (for early references see D'Ancona, 1923; Tiegs, 1924; van Harreveld, 1939).

Physiologically there is the problem whether the inhibitory axon exerts its effect on the motor terminals electrically, by current flow, or chemically,

by means of a transmitter. The motor endings appear to have chemoreceptor sites because the presynaptic neural inhibition could be reproduced by applying GABA. This substance mimics, in all known respects, the effects of the inhibitory transmitter (Kuffler, 1960). Another point in favour of chemical interaction is the timing relationship of impulses. The inhibitory impulse has to arrive near the junction more than 1 msec before the excitatory impulse for a maximal presynaptic inhibitory effect. Synaptic delays of 1 msec are usually seen in our nerve–muscle junctions. If the inhibitory transmitter acts on the excitatory nerve with the same delay, its effect would appear just at the time when the motor impulse arrives at that region. For an electrical interaction, on the other hand, a 1 msec minimal delay seems rather long. In addition, one can detect some presynaptic inhibition even when the inhibitory impulse precedes the excitatory one by 5–6 msec (Fig. 4). This fits much better if the interaction is chemical, and makes electrical transmission unlikely.

In respect to the mechanisms of the reduction of the probability of release by inhibition, very few facts are available. We have evidence that under normal conditions the nerve impulse, or perhaps its attenuated electrotonic extension, does get to the terminals even when no quanta are released (Dudel & Kuffler, 1961*b*). The chemical steps between the membrane potential change and the release of transmitter are unknown. Thus, when we measure the probability of release we are dealing with the end-product of an unknown chain of events. Therefore, any discussion about the control of transmitter release during facilitation or inhibition remains speculative (Curtis & Eccles, 1960). The principal facts for which any eventual theory will have to account are these: inhibition can reduce post-tetanic facilitation or the release of spontaneous miniature potentials only if the inhibitory impulses precede the excitatory ones by a critical time; and further, the inhibited e.j.p.s still fit the Poisson distribution curve, only the probability of release is reduced. Significantly, even during inhibition, the failure to release a quantum after a stimulus does not influence the average quantum content of the e.j.p. set up by the next stimulus. This excludes inhibitory action being an intermittent conduction block at such distance from the junction that no effective membrane potential change would reach the terminal.

Although many possible mechanisms might explain presynaptic inhibition, we would point out that the main known action of the inhibitory transmitter can be used for a consistent explanation. One may assume that the inhibitory nerve impulse releases in its terminal region a transmitter which acts on the neighbouring motor nerve fibre by increasing the conductance for a few milliseconds, as it does in the post-synaptic muscle membrane. The increased conductance, in turn, will decrease the electrical potential

change which serves as a 'stimulus' for the terminal. If this change were electrotonic (see below) one could explain the fact that the inhibitor reduces the e.j.p. independently of its size (Table 3). The size of the stimulus probably controls the average amount of quanta which are released and could also determine the extent of post-tetanic facilitation. That the size of a nerve impulse can control transmitter output has been suggested by the experiments of del Castillo & Katz (1954), who hyperpolarized the terminals and obtained larger end-plate potentials. Hagiwara & Tasaki (1958) also were able to reduce or increase synaptic potentials in the squid stellate ganglion, by changing the presynaptic membrane potential.

The advantage of the above suggestions is the possibility of an experimental test. Attempts to record changes in the size of the motor nerve impulse in the terminals have not yet been successful. We have also tried to determine the 'excitability' of the terminals (Wall, 1958) by stimulating them at the spots where the extracellular junctional potentials were recorded. It was not possible to set up impulses in the motor axon by passing current through this terminal area. This may lead one to suspect that the fine terminal stretch actually does not carry all-or-none impulses as the axon does, but is reached by electrotonic potentials only.

There are some suggestions for a presynaptic mechanism of inhibition elsewhere in the nervous system. Of special interest is the observation by Frank & Fuortes (1957), who saw inhibition of monosynaptic excitation in the spinal cord of cats without any appreciable change in the excitability of the post-synaptic cell. This 'remote inhibition' of Frank & Fuortes may well be presynaptic. A similar report comes from J. C. Eccles and his co-workers (personal communication). Therefore a definite possibility exists that the mammalian nervous system may also make use of a presynaptic mechanism.

SUMMARY

1. At crustacean neuromuscular junctions, as well as in numerous synapses elsewhere, the inhibitory transmitter acts by increasing the post-synaptic permeability to certain ions while the membrane potential remains near the resting level.

2. If inhibitory impulses are timed to arrive at the neuromuscular junction 1–6 msec before the excitatory impulse, they reduce the excitatory junctional potentials (e.j.p.s). It is demonstrated that in specific instances this reduction of e.j.p.s cannot be brought about solely by an increase in a post-synaptic inhibitory conductance.

3. The average number and size of quanta released by an excitatory nerve stimulus from nerve terminals were determined at individual junctional areas with extracellular micro-electrodes. Properly timed inhibition

reduced the number of released quanta while their size remained unchanged. Therefore, this type of inhibition acts at a presynaptic site on the excitatory nerve terminals, reducing their output of transmitter. These results do not support as mechanisms of inhibition either a post-synaptic decrease of sensitivity to the transmitter or a conduction block of the excitatory nerve.

4. Applied gamma-aminobutyric acid has an inhibitory effect in addition to its post-synaptic inhibitory conductance increase. This and a minimal synaptic delay of about 1 msec suggest chemical transmission from the inhibitory to the excitatory nerve terminals.

5. Presynaptic inhibition reduces the post-excitatory facilitation of e.j.p.s as well as the post-excitatory frequency increase of spontaneous miniature potentials. Presynaptic inhibition itself shows a process of facilitation, much like the inhibitory junctional potential.

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DRINKING BY NEPHRECTOMIZED RATS INJECTED WITH VARIOUS SUBSTANCES

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Gilman (1937), experimenting on normal dogs, observed that hypertonic solutions of sodium chloride, which cause cellular dehydration, gave rise to more drinking than hypertonic solutions of urea, which do not cause loss of cell water. He concluded that thirst is due to cellular dehydration.

Experiments on dogs by Holmes & Gregersen (1950*a*, *b*) in which a variety of substances were given, confirmed that osmotically effective solutes cause most drinking and by implication most thirst, and Adolph, Barker & Hoy (1954) showed that sodium chloride was twice as powerful as urea in causing normal rats to drink. In both species, however, no simple quantitative relationship was found between the amount of substance injected and the amount of water drunk.

In the present work it was thought that the analysis would be simplified if the kidneys were removed, because there could be no renal response to the substance, which could therefore act until neutralized by drinking, and the quantitative relation between the amount of substance injected and water drunk could thus be investigated. It was hoped that the cellular dehydration hypothesis of drinking would be tested in this way.

METHODS

Male albino rats, weighing between 150 and 400 g, were most commonly used in these experiments, but some female rats and a small number of hooded rats and ginger rats of both sexes were used also. Food (Diet 41, Bruce & Parkes, 1949) was available until the beginning of the experiment and water was available before and during the experiment from a drinking meter which enabled the time course of drinking to be recorded (Fitzsimons, 1958).

Experimental procedure

Each animal was anaesthetized with open ether and the external jugular vein catheterized by a soft polyvinyl chloride tube, outside diameter 1 mm. The tip of the catheter was advanced to the right atrium to minimize the complication of dead space when injections were being made. The catheter was brought subcutaneously to the back of the neck and about 2 cm was left projecting through a stab wound in the skin. The catheter, which was filled with 0·9 % sodium chloride solution, was closed with a small plug.

Bilateral nephrectomy was then carried out through a dorsal incision. A clean surgical technique was employed, but no aseptic precautions were taken because the experiments

only lasted for 6 hr. In some early experiments unilateral nephrectomy was performed a few days previously and at the same operation a loose ligature was placed round the pedicle of the remaining kidney and the ends taken out through the skin wound. This ligature was tied just before the start of the experiment with little apparent discomfort to the animal, completing destruction of renal function. The results obtained after the two methods of nephrectomy were the same.

About 1–1½ hr after the operation a known amount of solute in hypertonic solution was injected through the jugular catheter, washing through with 0·9% sodium chloride solution so that the total injection volume was approximately 1 ml./100 g body weight. After large doses of the less soluble substances this percentage was exceeded. During the injection the rat was not restrained, but allowed to walk around if it wished. If there were signs of discomfort, e.g. head movements, the rate of injection was slowed.

After the injection, the rat was weighed to the nearest 0·1 g and put into an individual metabolism cage. Spontaneous drinking was recorded for the next 6 hr. As will be seen later, 6 hr was usually long enough for the rat to recover from the injection and finish drinking. At the end of 6 hr the rat was weighed, together with any faeces passed; water loss from faeces after voiding was regarded as part of the non-urinary water loss of the animal. The majority of the rats did not in fact defaecate, and those that did passed one or two pellets only.

Control rats were subjected to the same procedures as the experimental animals except that they were given 1 ml./100 g body weight 0·9% sodium chloride solution intravenously.

In some experiments the inulin space was measured by injecting 1 ml. of 10% inulin (Kerfoot) intravenously at the time of nephrectomy and estimating the serum concentration at the end of the experiment by a resorcinol method (Bell, 1955).

In some experiments also the serum freezing points were measured thermoelectrically at the end of the 6 hr period.

Solutions

Solutions for injection were prepared from Analar reagents dissolved in 0·9% sodium chloride solution, except sodium chloride itself which was dissolved in water (Table 1). Sugar and urea solutions were freshly prepared; electrolyte solutions were stored in glass-stoppered bottles. The solutions were not sterilized.

Table 1. Concentration (M) of substances in solutions: each substance, except NaCl, was dissolved in isotonic NaCl solution (9 g/l. water)

Electrolytes		Non-electrolytes	
NaCl (in water)	2	Glucose	2
CH_3COONa	2	Methyl glucose	2
Na_2SO_4	2*	Fructose	2
KCl	1	Sucrose	2
CH_3COOK	1	Sorbitol	2
		Mannitol	2*
		Urea	8

* Only dissolves completely when warm.

Expression of results

The weight changes of the whole animal were used as measures of water balance, ignoring respiratory weight changes, metabolic water and water remaining in the alimentary tract. In 6-hr experiments on rats not permitted access to food these amounts are small (Jelleff, Carr & Krantz, 1942), tend to cancel one another, and are presumably approximately the same for control and experimental groups.

The water balance of the animal, i.e. gain or loss of weight, is expressed as a percentage of the initial body water of the animal, which includes the volume of fluid injected. Initial

body water was estimated on 91 rats by subtracting weight changes from final body water measured by evaporating the comminuted carcass to constant weight at 100–105° C. The observed mean of 69 g/100 g initial body weight has been used in all calculations.

The amount of hypertonic solution injected is given in ml./100 g initial body weight, where initial body weight is the post-injection body weight. The amount of solution injected is also expressed in terms of the percentage increase in osmotic pressure of body fluids. To avoid having to restrain and bleed the experimental group, which might affect drinking, the increases in osmotic pressure were not measured directly, but were calculated by using osmotic coefficients appropriate to the ionic strength of the mixture of body fluid (cf. Conway's 1957 data for the rat) and injected substance (Robinson & Stokes, 1955; Bayliss, 1959). The validity of the procedure was confirmed by measuring the freezing points of mixtures of sodium chloride and sodium sulphate. The coefficients of 0·96 for sodium and potassium acetate, 0·93 for sodium chloride and 0·92 for potassium chloride were taken from the International Critical Tables (1928). The sodium sulphate coefficient, which varied between 0·805 and 0·825 depending on the amount of sodium sulphate injected, was taken from the data of Robinson & Stokes (1955). For all non-electrolytes a coefficient of 1 was assumed.

An example of the calculation when sodium sulphate was injected is as follows. A group of rats were given 0·488 ml. 2 M sodium sulphate/100 g initial body weight. Assuming that the sodium sulphate remains outside the cells, and that the cells behave as osmometers, the approximate ionic strength of the mixture of extracellular fluid and sodium sulphate is 0·24 and the appropriate osmotic coefficient is 0·81. The amount of sodium sulphate added was therefore $0{\cdot}488 \times 6 \times 0{\cdot}81 = 2{\cdot}372$ m-osmole/100 g initial body weight. The real osmolality of body fluids before the injection was found to be 290 m-osmole/kg H_2O, thus the initial osmotic content of the whole body fluids was $69 \times 290/1000 = 20$ m-osmole/100 g initial body weight. The increase in osmotic pressure was therefore approximately 11·9 %. The calculations for the other substances are essentially the same, except that when sodium chloride was given allowance was made for the salt needed to bring the hypertonic volume to isotonicity; other hypertonic solutions were made up in isotonic sodium chloride.

RESULTS

Description of a typical experiment

The behaviour of nephrectomized rats for the first 24 hr after operation was not obviously different from normal. Food and water were still taken though consumption of both fell. Intake of water was sufficient to balance losses for at least the first 24 hr after nephrectomy.

In 30 control rats which had received isotonic sodium chloride solution (Table 2) the average volume of water drunk was 1·82 ml./100 g initial body water and the average weight loss was 0·17 g/100 g initial body water. Change in weight shows the water balance (see under Methods): the controls thus effectively maintained water balance, and the deviations of water balance of the experimental animals from zero may therefore be attributed to the injected solutions.

A kymograph record of drinking by a nephrectomized rat injected with 0·327 ml. 2 M sodium sulphate/100 g initial body weight is given in Fig. 1. The increase in osmotic pressure produced by this dose was 8 %, the fluid balance after 6 hr was 10·8 g/100 g initial body water and the amount of water drunk was 13·5 ml./100 g initial body water.

The drinking record of Fig. 1 is quite typical of records obtained after injection of a variety of substances, in that most drinking occurred in the first 2 hr. This is further illustrated in Fig. 2, which gives the mean rates of drinking after all the doses of sodium sulphate used. After most doses

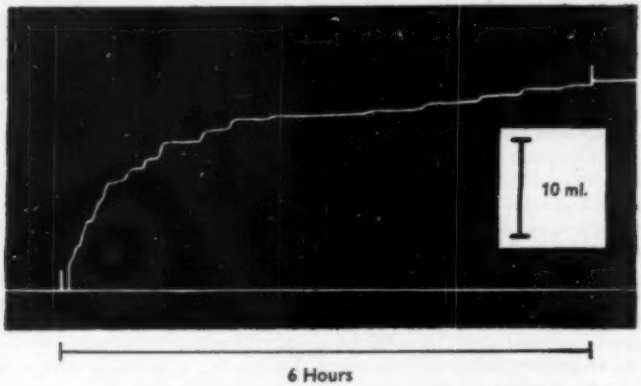

Fig. 1. A kymograph record of drinking by a nephrectomized rat after injection of 0·327 ml. 2M sodium sulphate/100 g body weight.

TABLE 2. The amount of water drunk, fluid balance, inulin space and serum freezing point of nephrectomized rats 6 hr after injection of various doses of sodium chloride

2M NaCl injected (ml./100 g initial body wt.)	Increase in initial osmotic pressure (%)	Water drunk (ml./100 g initial body H_2O)	Fluid balance (g/100 g initial body H_2O)	Inulin space (ml./100 g initial body H_2O)	Difference between control and experimental inulin spaces	Serum freezing point at 6 hr (°C)
Control	0	1·82	−0·17 ± 0·27 (30)	35·52 ± 0·99 (10)	—	−0·566 ± 0·0007 (10)
0·086	1·5	3·5	1·13 ± 0·81 (5)	—	—	—
0·162	2·8	4·47	2·72 ± 0·95 (5)	—	—	—
0·272	4·7	6·36	4·64 ± 0·55 (12)	40·16 ± 1·09 (10)	4·64	−0·562 ± 0·0079 (10)
0·364	6·2	7·17	5·7 ± 0·28 (3)	—	—	—
0·536	9·2	9·17	7·4 ± 0·5 (15)	43·86 ± 1·24 (10)	8·34	—
0·805	13·8	10·98	8·95 ± 0·64 (10)	47·0 ± 1·42 (9)	11·48	−0·585 ± 0·0088 (9)
1·042	17·8	10·09	8·13 ± 1·89 (5)	45·24 ± 2·13 (5)	9·72	−0·636 ± 0·0114 (5)

In this and subsequent tables the mean ± S.E. is given, with the number of observations in parentheses

there was a phase of rapid drinking lasting about 2 hr, followed by a phase in which the rate of drinking was only slightly more than the control rate. After the highest dose the initial rate of drinking was much slowed because of incapacitating tetany that sodium sulphate causes in these doses. It would seem from these results that 6 hr is ample time for a nephrectomized rat to achieve equilibrium after injections which cause drinking.

A comparison of drinking by normal and nephrectomized rats

The amounts of water drunk by normal and nephrectomized rats after various doses of sodium chloride or urea are plotted in Fig. 3. After the smaller doses of both substances the nephrectomized animals drank more water than the normal animals. After the higher doses of urea the position is reversed and the normal animals drank more than the nephrectomized, probably because of the loss of fluid due to the osmotic diuresis. From the trend of the curves in Fig. 3*a*, the same would almost certainly hold after sodium chloride if larger doses had been used.

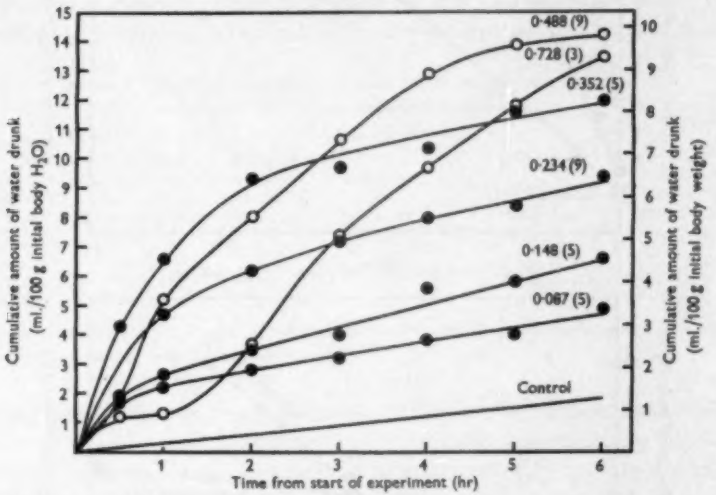

Fig. 2. Cumulative amounts of water drunk after various doses of 2M sodium sulphate. Amount of sodium sulphate injected (ml./100 g body weight) and number of animals used are given on each curve.

Drinking caused by sodium chloride in nephrectomized rats

The amounts of water drunk, the fluid balances, inulin spaces and serum freezing points of nephrectomized rats 6 hr after injection of various doses of sodium chloride are given in Table 2. The doses of sodium chloride are also expressed in terms of increases in initial osmolality. The mean initial serum freezing point of 12 control rats was $-0{\cdot}540 \pm 0{\cdot}0061°$ C (S.E. of mean), giving a real osmolal concentration of *ca.* 290 m-osmole/kg H_2O, and the percentage increases were calculated in the way described above.

When isotonicity is restored by drinking there is numerical equality between the percentage increase in initial osmotic pressure and the fluid balance expressed as a percentage of initial body water. Doses of 2M

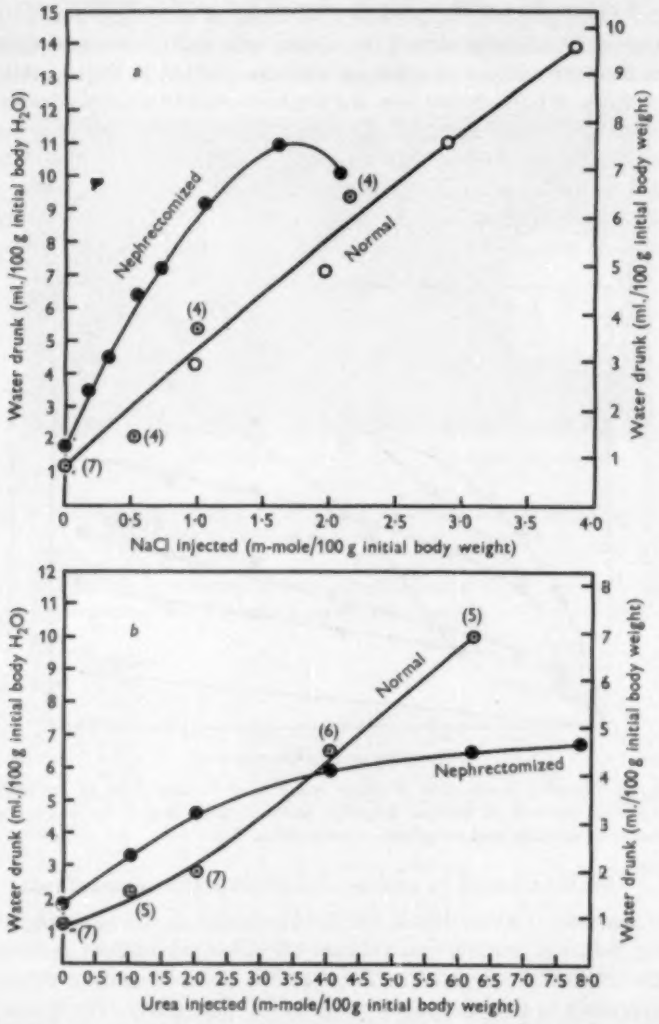

Fig. 3. The amounts of water drunk by normal and nephrectomized rats after various doses of (*a*) sodium chloride, and (*b*) urea. The data for nephrectomized rats plotted as ● are taken from Tables 2 and 7; data for normal rats plotted as ⊙ were obtained in experiments similar to those on nephrectomized animals, described in this paper; those plotted as ○ are calculated from the data of Adolph, Barker & Hoy (1954).

sodium chloride solution up to 0·272 ml./100 g initial body weight, equivalent to a 4·7% increase in initial osmotic pressure, were diluted to isotonicity by drinking. After higher doses net fluid intake was inadequate to restore osmotic pressure to normal. The serum freezing points confirm this conclusion.

Hypertonic sodium chloride solution produces changes similar to those produced by water deprivation, namely, cellular dehydration and an over-all increase in osmotic pressure of body fluids, without presumably any very marked change in the relative chemical composition of body fluids. One important difference, especially in the nephrectomized rat, is that sodium chloride causes expansion of the extracellular space. This expansion becomes more marked as the nephrectomized animal drinks, and

TABLE 3. The effect of expanding body fluids on drinking induced by hypertonic NaCl in nephrectomized rats

Isotonic NaCl infused (ml./100 g initial body H_2O)	2 M-NaCl injected (ml./100 g post-infusion body wt.)	Increase in initial osmotic pressure (%)	Water drunk (ml./100 g. post-infusion body H_2O)	Fluid balance (g/100 g post-infusion body H_2O)	Fluid balance (g/100 g pre-infusion body H_2O)
0	0·805	13·8	10·98	8·95 ± 0·64 (10)	8·95
3·09	0·819	13·8	10·3	8·98 ± 0·92 (6)	12·8
10·03	0·824	13·6	10·24	8·16 ± 1·33 (9)	20·5

Proportionately larger doses of hypertonic NaCl were given to the infused animals to allow for the greater initial body water.

it may be seen that in the experiments of Table 2 the differences between the control and experimental inulin spaces were approximately equal to the fluid balances, i.e. the net fluid intakes were mostly accommodated in the extracellular spaces.

To determine whether expansion of extracellular space affects drinking, experiments were carried out in which injections of hypertonic sodium chloride solution were immediately preceded by large infusions of isotonic sodium chloride solution, given over a period of about 5 min. It was found that the response to hypertonic sodium chloride, calculated in terms of the augmented initial body water, was the same as for the group which had not been previously infused (Table 3). This occurred despite a fluid balance, including the isotonic infusion, considerably in excess of the largest fluid balance produced by a drinking stimulus alone. Thus this degree of expansion of extracellular fluid is not a factor modifying drinking.

The nephrectomized rat is a suitable preparation to study adaptation of a thirst receptor, because once sodium chloride is injected it must remain in the animal. If drinking is not allowed until some hours after the injection, any adaptation should be revealed by comparing the fluid balance

with the fluid balance when drinking was permitted from the start of the experiment.

It was found (Table 4) that withholding water for 2 hr after injection of hypertonic sodium chloride solution had little effect on the final fluid balance in a 6 hr experiment. When water was withheld for 4 hr, so that the time available for drinking was reduced to 2 hr, net intake of water was a little less, but records of the rate of drinking show that this was because there was less time for drinking rather than adaptation (Fig. 4).

Drinking caused by sodium sulphate, sodium acetate and sucrose in nephrectomized rats

Data for the effects of sodium sulphate, sodium acetate and sucrose in nephrectomized rats are given in Table 5. Sodium sulphate was found to

TABLE 4. The effect of temporarily withholding drinking water after administration of hypertonic NaCl

Duration of water deprivation (min)	2 M-NaCl injected (ml./100 g initial body wt.)	Increase in initial osmotic pressure (%)	Fluid balance (g/100 g initial body H_2O)
0	0·536	9·2	7·4 ± 0·5 (15)
120	0·523	9·0	7·4 ± 1·15 (6)
240	0·541	9·25	6·64 ± 0·73 (6)

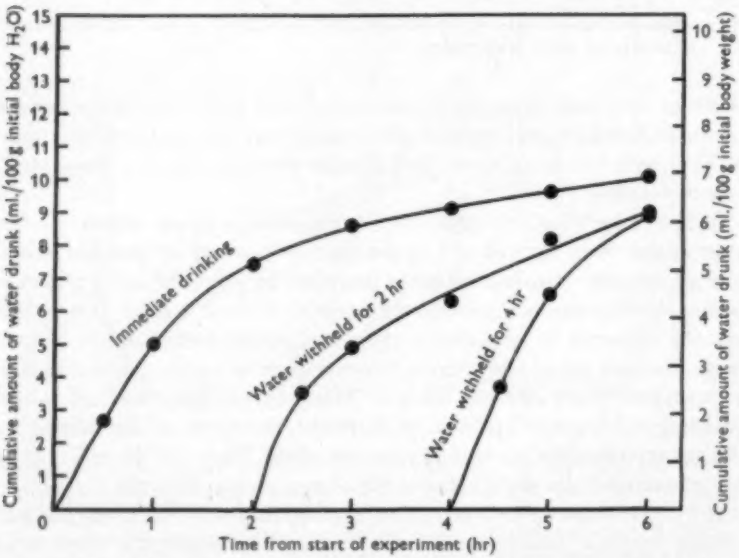

Fig. 4. The effect of temporarily withholding water after injection of hypertonic sodium chloride on the subsequent rates of drinking (see Table 4).

cause the most drinking of all substances tested. Fluid balances were more than sufficient to neutralize up to about 0·49 ml. 2 M sodium sulphate/100 g initial body weight, equivalent to a 12% increase in initial osmotic pressure. The serum freezing points confirm that the animals tended to drink to hypotonicity after the lower doses of sodium sulphate, and as after sodium chloride the inulin spaces show that the net fluid intake was mostly accommodated in the extracellular space.

TABLE 5. The amount of water drunk, fluid balance, inulin space and serum freezing point of nephrectomized rats 6 hr after injection of various doses of hypertonic sodium sulphate sodium acetate or sucrose

Volume of hypertonic solution injected (ml./100 g initial body wt.)	Increase in initial osmotic pressure (%)	Water drunk (ml./100 g initial body H_2O)	Fluid balance (g/100 g initial body H_2O)	Inulin space (ml./100 g initial body H_2O)	Difference between control and experimental inulin spaces	Serum freezing point at 6 hr (°C)
Control	0	1·82	−0·17 ± 0·27 (30)	35·52 ± 0·99 (10)	—	−0·566 ± 0·0007 (10)
2 M-Na_2SO_4						
0·087	2·2	4·92	2·59 ± 0·25 (5)	36·96 ± 0·39 (5)	1·44	—
0·148	3·7	6·58	4·32 ± 0·56 (5)	—	—	—
0·234	5·8	9·1	6·77 ± 0·78 (9)	44·82 ± 0·37 (7)	9·3	−0·553 ± 0·0078 (8)
0·352	8·6	12·0	9·63 ± 1·02 (9)	—	—	—
0·488	11·9	14·24	12·44 ± 1·34 (9)	51·3 ± 1·81 (9)	15·78	−0·556 ± 0·0044 (9)
0·722	17·5	15·24	12·4 ± 1·68 (6)	48·4 ± 1·82 (6)	12·88	−0·598 ± 0·0043 (6)
2 M-CH_3COONa						
0·149	2·8	4·6	2·61 ± 0·04 (5)	—	—	—
0·243	4·7	5·92	3·99 ± 0·9 (5)	—	—	—
0·496	9·5	8·45	6·67 ± 0·54 (10)	—	—	—
0·745	14·3	10·24	8·34 ± 1·17 (9)	43·92 ± 0·67 (8)	8·4	—
2 M Sucrose						
0·159	1·6	2·42	1·36 ± 0·8 (3)	—	—	—
0·335	3·3	5·5	3·29 ± 0·35 (3)	—	—	—
0·536	5·4	6·35	4·8 ± 1·29 (3)	—	—	—
0·738	7·4	7·5	5·8 ± 0·31 (3)	—	—	—
1·055	10·5	8·21	6·59 ± 0·81 (6)	—	—	—
1·58	15·8	10·09	8·35 ± 0·58 (8)	—	—	—

In some animals the larger doses of sodium sulphate caused overt tetany. On two occasions the experiment had to be stopped because the rat was distressed, but usually the rat recovered quite quickly and would start or continue to drink. The mean delays before the onset of drinking after the highest doses of sodium chloride and sodium sulphate show the effect of tetany (Table 6), as do the curves of the rates of drinking in Fig. 2.

The response to sodium acetate (Table 5) was almost the same as to sodium chloride, perhaps surprisingly in view of the fact that acetate is metabolized. However, the presence of sodium ions in the extracellular space requires anions for electrical neutrality, presumably bicarbonate, which subserve the same osmotic function as acetate. It has been shown

that sodium acetate causes an alkalosis in no way different from a bicarbonate alkalosis (Lipsky, Alper, Rubin, Van Eck & Gordon, 1954). Sucrose (Table 5) was as effective as sodium chloride and sodium acetate in causing drinking. The close agreement between the fluid balances after doses of equal osmotic strength is striking.

TABLE 6. Intervals before the onset of drinking after the higher doses of sodium chloride and sodium sulphate

NaCl			Na_2SO_4		
2 M-NaCl injected (ml./100 g initial body wt.)	Increase in initial osmotic pressure (%)	Mean latency (min)	Mean latency (min)	Increase in initial osmotic pressure (%)	2 M-Na_2SO_4 injected (ml./100 g initial body wt.)
0·805	13·8	7·3 (10)	40 (9)	11·9	0·488
1·042	17·8	10 (5)	107 (6)	17·5	0·722

TABLE 7. The amount of water drunk and fluid balance of nephrectomized rats 6 hr after injection of substances which did not cause much drinking, and the effect of temporarily withholding drinking water after injection of urea

Volume of hypertonic solution injected (ml./100 g initial body wt.)	Increase in initial osmotic pressure (%)	Water drunk (ml./100 g initial body H_2O)	Fluid balance (g/100 g initial body H_2O)
Control	0	1·82	−0·17 ± 0·27 (30)
2 M Sorbitol			
0·532	5·3	3·5	1·98 ± 0·38 (6)
1·04	10·4	4·9	2·89 ± 0·42 (5)
1·55	15·5	5·83	3·2 ± 0·88 (7)
2M Mannitol			
1·52	15·2	3·5	2·01 ± 1·36 (5)
2M Glucose			
1·575	15·75	2·4	0·27 ± 0·43 (15)
2M Methyl glucose			
1·59	15·9	2·47	0·79 ± 0·66 (5)
2M Fructose			
1·573	15·7	4·13	2·09 ± 0·62 (10)
8M Urea (immediate access to drinking water)			
0·127	5·1	3·3	0·98 ± 0·62 (10)
0·253	10·1	4·62	2·34 ± 0·49 (8)
0·505	20·2	5·86	3·44 ± 0·65 (8)
0·775	31·0	6·49	4·25 ± 0·38 (9)
0·98	39·2	6·75	4·59 ± 0·96 (5)
8M Urea (drinking water withheld for 165–230 min after injection)			
0·752	30·1	5·6	3·7 ± 0·61 (8)

Substances which did not cause much drinking

The effects of sorbitol, mannitol, glucose, methyl glucose, fructose and urea on the water intake of nephrectomized rats are given in Table 7. In this class, sorbitol, mannitol, fructose and urea were the most effective and glucose and methyl glucose the least effective. Fluid balances after glucose and methyl glucose were only slightly greater than the control

balance. Urea had a definite effect on drinking, but even after the very large dose of 0·98 ml. 8M urea/100 g initial body weight, giving an increase in osmotic pressure of about 39%, the positive fluid balance was only 4·6 g/100 g initial body water. Delayed access to drinking water after urea administration did not markedly alter the amount of water drunk subsequently.

TABLE 8. The amount of water drunk and fluid balance of nephrectomized rats 6 hr after injection of potassium salts, and the effect of temporarily withholding drinking water after injection of potassium chloride

Volume of hypertonic solution injected (ml./100 g initial body wt.)	Increase in initial osmotic pressure (%)	Water drunk (ml./100 g initial body H_2O)	Fluid balance (g/100 g initial body H_2O)
Control	0	1·82	$-0·17 \pm 0·27$ (30)
1M-CH_3COOK			
0·344	3·3	3·13	$1·94 \pm 0·19$ (5)
1M-KCl (immediate access to drinking water			
0·25	2·3	3·5	$1·5 \pm 0·54$ (5)
0·413	3·8	4·5	$2·31 \pm 0·86$ (5)
0·621	5·7	5·84	$4·07 \pm 1·09$ (5)
1M-KCl (drinking water withheld for 130–230 min after injection)			
0·618	5·7	5·12	$3·14 \pm 0·67$ (10)

Drinking caused by potassium salts in nephrectomized rats

Potassium salts were unexpectedly effective in causing nephrectomized rats to drink, even when immediate access to drinking water was prevented (Table 8). Potassium salts, particularly in the large doses used here, are very toxic. Injections were given slowly in divided doses and part of the dose was often given subcutaneously, but despite these precautions toxic manifestations were the rule. These consisted of irregular movements of muscle including the respiratory muscles, temporary paralysis and irregular action of the heart. There was apparently complete recovery 5–10 min later and the rats would start to drink.

DISCUSSION

A number of substances cause nephrectomized rats to drink, casting doubt on the assertion that the sensation of thirst does not occur in the nephrectomized rat (Linazasoro, Jiménez Diáz & Castro Mendoza, 1954). Sodium chloride, sodium sulphate, sodium acetate and sucrose were the most effective substances in causing drinking and were equally effective in isosmotic doses when the doses were small. It is therefore unlikely that drinking was due to specific chemical properties of the substances, but rather it was due to a common or colligative property such as osmotic pressure. Furthermore, these are substances which are known to dehydrate most of the cells in the body. In doses producing increases of up to 5% in

osmotic pressure, comparable with the increases met in normal life, these substances caused enough drinking to restore the osmotic pressure of body fluids to normal, as is shown in Fig. 5 where the line $0A$ represents restoration of isotonicity. However, restoration of body fluids to isotonicity is quite incidental as is shown by the relative failure of substances such as urea and glucose, which penetrate cells, to cause drinking. In the case of

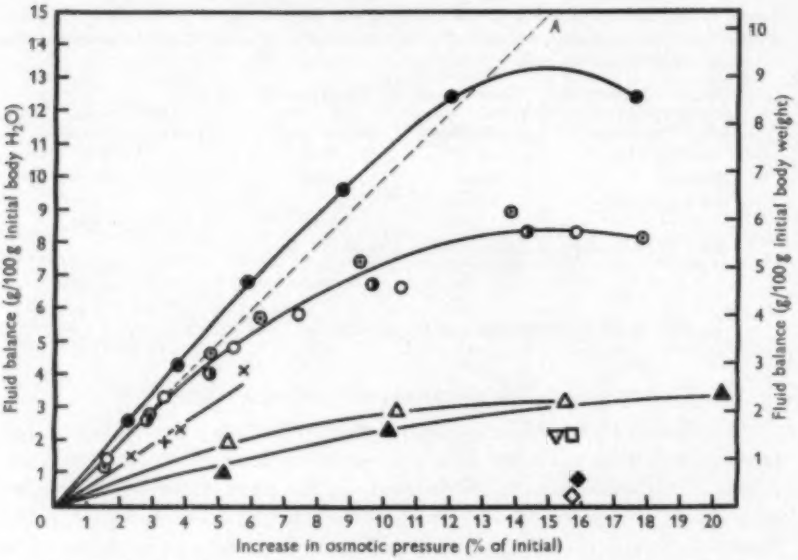
A
Fluid balance (g/100 g initial body H_2O)
Fluid balance (g/100 g initial body weight)
Increase in osmotic pressure (% of initial)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
1 2 3 4 5 6 7 8 9 10

Fig. 5. The relationship between increase in initial osmotic pressure produced by injection of various substances and the net fluid intake of nephrectomized rats. The data are taken from Tables 2, 5, 7 and 8. The line $0A$ delineates the theoretical relationship predicted for an osmotically effective substance by a simple cellular dehydration hypothesis of drinking, indicating restoration of cell size. ● Na_2SO_4, ○ sucrose, ⊙ NaCl, ◑ CH_3COONa, × KCl, + CH_3COOK, △ sorbitol, ▲ urea, ▽ mannitol, □ fructose, ◇ glucose, ◆ methyl glucose.

injected substances which are confined to the extracellular space, sufficient drinking to dilute these substances to isotonicity also restores the cells to their initial size, provided there is no net movement of normal body fluid constituents across the cell membranes. If it be assumed that the cells in a thirst receptor zone behave in the same way as the majority of cells in the body, then as a first approximation these results are in accordance with a hypothesis that thirst and drinking are due to dehydration of cells in a receptor zone.

Further support for the hypothesis is provided by the inulin spaces,

which show the lack of appreciable cellular dehydration after the higher and unneutralized doses of sodium chloride, sodium sulphate and sodium acetate when the animals had voluntarily stopped drinking. The animals had stopped drinking when presumably the receptor cells had been restored to their normal size, even though the injected substances had not been diluted to isotonicity.

Holmes & Gregersen (1950*a*) and Adolph *et al.* (1954) emphasized that in intact animals the amount of water drunk in response to an osmotic stimulus is not simply related to the amount of water required to restore the animal to isotonicity, and that if drinking were not permitted during the first few hours after the injection, the amount of water subsequently drunk was the same as when drinking was allowed immediately after the injection, even though the injected substance had been mostly excreted in this time.

One reason for their failure to find a simple relationship between the amount of substance injected and drinking is that the experiments were carried out on animals with functioning kidneys. Adolph and his colleagues in fact questioned the view that drinking may be modified by a renal response to the injected substance, saying, 'we found no good evidence that drinking was stimulated as an alternative to the performance of osmotic work in the kidneys.' The present experiments provide such evidence, because it was found that nephrectomized animals drank more water than normal animals after identical injections. It seems reasonable to conclude that the extra amount of water was drunk because the injected substances were not removed by renal excretion.

Another complication is that Adolph *et al.* (1954) used oral and intraperitoneal routes for administration of hypertonic urea or sodium chloride. The intraperitoneal injection of hypertonic solutions appears to be painful, and moreover, there may be temporary pooling of fluid in the peritoneal cavity, so that any effect on drinking may be secondary. These authors in fact found different responses to the same amount of solute given by the two routes. The advantages of intravenous injections are that they cause least apparent discomfort to the animals, they avoid the complication of pooling and they allow rapid distribution of the injected substance.

A third reason is that all previous workers measured the amount of water drunk rather than the net fluid intake. In a rapid drinker like the dog this may be reasonable, though there is the complication of temporary gastro-pharyngeal satiation (Montgomery & Holmes, 1955). In a slow drinker such as the rat, however, non-urinary loss of water during the drinking period will appreciably alter the amount of water available for diluting the injected substance.

Though after lower doses of sodium sulphate, sodium chloride, sodium acetate and sucrose, fluid balances were close to the values expected on

a cellular dehydration hypothesis of drinking, after higher doses the response curves fell away from the predicted relationship (Fig. 5). Expansion of extracellular fluid does not appear to be a factor, because in the experiments in Table 3 infusion of isotonic sodium chloride did not modify the drinking caused by subsequent injection of hypertonic sodium chloride. The total fluid balance of these animals, including the infusion, was considerably greater than the largest fluid balance achieved by drinking alone. Nor does adaptation of the receptor appear to be important, because a 4 hr delay had little effect on the response to sodium chloride (Table 4). The serum freezing-point values rule out the possibility that the injected substances were sequestered in osmotically inactive forms, yet from the inulin spaces large doses of these substances appear to be osmotically ineffective. Endogenous release of cellular osmotic material raising the cellular osmotic pressure is a possible explanation of reduced osmotic effectiveness (McDowell, Wolf & Steer, 1955), but a more likely explanation is that the injected substances in large doses penetrate the cells. In the case of sucrose, reduced osmotic effectiveness could be due to metabolism. White & Rolf (1957) found that when inulin or sucrose was given to nephrectomized rats 100% of inulin was recoverable 6 hr later, but only 85·2% of sucrose.

Glucose and methyl glucose caused very little drinking, fructose rather more; all are freely permeant and would be expected to be ineffective on a cellular dehydration hypothesis. Urea did cause some drinking (Fig. 5). It has been found to cause some drinking in normal animals, but most authors assume that this is due to osmotic diuresis (e.g. Strauss, 1957), though Adolph *et al.* (1954) said that, 'only in time did drinking appear to dictate excreting rather than vice versa', implying that urea stimulated a thirst receptor directly. According to most authors urea should be without effect in nephrectomized animals, but it proved a more powerful stimulus of drinking than was expected, and significantly, in lower doses, more effective in nephrectomized animals than in normal animals. Transient osmotic stimulation of the receptor is ruled out by the experiments in which access to drinking water was delayed for some time after injection of the urea. The reason may be that the receptor itself is impermeable to urea. Conway & Fitzgerald (1942) found that there are regions in the central nervous system which are relatively impermeable to urea, and recently Javid & Anderson (1959) showed that hypertonic urea solutions have a powerful action in lowering cerebrospinal fluid pressure, the effect being greater and longer lasting after bilateral nephrectomy. It is interesting to note that Zuidema, Clarke & Minton (1956) obtained antidiuretic responses from lightly anaesthetized dogs with urea infusions which raised the osmotic pressure by 2% over a period of 40 min, though

Verney's (1954) results on conscious dogs show that the antidiuretic receptor is normally permeable to urea.

The sugar alcohols, sorbitol and mannitol, were about equally effective as urea in causing nephrectomized rats to drink. Holmes & Gregersen (1950*a*) on the contrary found that sorbitol given to normal dogs was as powerful a stimulus of drinking as sodium chloride, sodium sulphate and sucrose. Holmes & Gregersen state that sorbitol causes the same fluid shifts in the body as sodium chloride, but from their figures for plasma protein levels before and after injection of sodium chloride or sorbitol it is evident that the fluid shifts caused by sorbitol were more evanescent. This may be because sorbitol is metabolized (Blakley, 1951; mannitol is also metabolized, Hamburger & Mathé, 1952), or because it is more permeant than sodium chloride. Sorbitol would thus cause less drinking in the rat, a slow drinker, than in the dog, a rapid drinker. Apart from possible differences due to different rates of drinking, another reason for the discrepancy is that sorbitol is a powerful diuretic. The large amount of water drunk by normal dogs may therefore be due to renal loss of fluid.

The part played by potassium in drinking is interesting. The association between potassium deficiency and thirst seems well established (Black, 1957; Stanbury, 1958), and ingestion of potassium salts does not cause thirst or drinking according to Arden (1934) and Janssen (1936). These results are consistent with the cellular dehydration hypothesis, because loss of potassium might be expected to lead to cellular shrinkage, and administration of potassium should cause no alteration in cell size because most cells are freely permeable to potassium. The quite vigorous drinking caused by potassium salts was therefore an unexpected finding. Whether this was a non-specific response to stress, whether the receptor itself is impermeable to potassium or whether there is some other explanation, it is impossible to say on present evidence. The toxicity of extracellular potassium complicates the interpretation of results which depend on the voluntary response of the animal.

Sodium sulphate caused more drinking than any other substance (Fig. 5). There was, moreover, a tendency after the lower doses for the animals to drink more than was required for restoration of isotonicity. The reason for this overshoot is not known. One possibility is that the loss of cell water and dilution of normal extracellular constituents upsets the Donnan equilibrium. If enough potassium chloride were to leave the cells to restore the Donnan equilibrium and the animal drank sufficient water to achieve isotonicity, the cells would be slightly smaller than initially. To restore the cells to their initial size would require a slightly greater water intake which would make the body fluids hypotonic. The order of overshoot expected, calculated from Conway's (1957) data for the rat and assuming that potassium chloride is the only solute to cross

the cell membranes, agrees closely with the sodium sulphate response curve. The fact that sodium sulphate caused more drinking than any other substance supports the cellular dehydration hypothesis because sodium sulphate is more rigorously excluded from the cells than the other substances. The fact that the sodium sulphate response tends to overshoot the isotonic line might be regarded as additional evidence that it is the size of the cells in the receptor that determines whether drinking takes place. It should be emphasized in conclusion that cellular dehydration is not the only mechanism controlling water intake.

SUMMARY

1. Nephrectomized rats were found to drink more water than normal rats when given varying amounts of hypertonic sodium chloride solution or hypertonic urea solution, which produced increases of up to 15% in the osmotic pressure of body fluids.

2. Sodium sulphate caused most drinking in nephrectomized rats, but sodium chloride, sodium acetate and sucrose were almost as effective in doses producing up to 5% increase in osmotic pressure. The relationship between net fluid intake and increase in osmotic pressure after these substances was as predicted by a cellular dehydration or osmometer hypothesis of drinking, in that the animals generally stopped drinking when the cells were restored to their initial size.

3. A preliminary 10% isotonic expansion of body fluids did not prevent the full response to hypertonic sodium chloride.

4. Delayed access to drinking water after hypertonic sodium chloride did not significantly affect the amount of water drunk subsequently, nor the initial rate of drinking.

5. Potassium chloride and potassium acetate caused almost as much drinking in nephrectomized rats as the four most effective substances. Delayed access to drinking water after injection of hypertonic potassium chloride did not affect the amount of water drunk subsequently.

6. Urea, sorbitol, mannitol and fructose were considerably less effective but nevertheless caused appreciable drinking. Delayed access to drinking water after injection of hypertonic urea did not cause much reduction in the amount of water drunk subsequently. Glucose and methyl glucose were the least effective substances tested, and net fluid intakes were only slightly greater than control intakes.

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KINETICS OF THE REACTIONS OF TWO SHEEP HAEMOGLOBINS WITH OXYGEN AND CARBON MONOXIDE

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The kinetics of the reactions of haemoglobin with oxygen, carbon monoxide and other ligands have been extensively studied in recent years. The basis of these studies has been the intermediate-compound hypothesis due to Adair (1925). According to this hypothesis the combination of haemoglobin with oxygen may be represented by the four consecutive reactions

$$Hb_4 + O_2 \underset{k_1}{\overset{k_1'}{\rightleftharpoons}} Hb_4O_2; \quad \frac{k_1'}{k_1} = K_1,$$

$$Hb_4O_2 + O_2 \underset{k_2}{\overset{k_2'}{\rightleftharpoons}} Hb_4O_4; \quad \frac{k_2'}{k_2} = K_2,$$

$$Hb_4O_4 + O_2 \underset{k_3}{\overset{k_3'}{\rightleftharpoons}} Hb_4O_6; \quad \frac{k_3'}{k_3} = K_3,$$

$$Hb_4O_6 + O_2 \underset{k_4}{\overset{k_4'}{\rightleftharpoons}} Hb_4O_8; \quad \frac{k_4'}{k_4} = K_4.$$

Estimates of K_1, K_2, K_3, and K_4 may be obtained from accurate experimental observations on the equilibrium with oxygen. These observations must not only cover the range of saturation usually studied (5–95 % saturation) but must also include specially accurate observations between 0 and 1 % saturation and between 99 and 100 % saturation.

Roughton, Otis & Lyster (1955) describe how the necessary observations may be made and how the values of K_1, K_2, K_3, and K_4 may be derived therefrom, together with their standard errors. They give actual sets of the constants for several samples of sheep haemoglobin, under the standard conditions of 19° C and pH 9·1.

The reactions of haemoglobin with carbon monoxide may be represented

in a similar way to those with oxygen. In this case the symbols used for equilibrium and kinetic constants are

$$L_1 = \frac{l'_1}{l_1}, \quad L_2 = \frac{l'_2}{l_2}, \text{ etc.}$$

The four velocity constants l'_1, l'_2, l'_3 and l'_4 are sufficient to describe the major part of the course of the over-all reaction between reduced haemoglobin and carbon monoxide, as the rate of dissociation of combined carbon monoxide may be neglected. Values of these constants for several samples of sheep haemoglobin have been determined by Gibson & Roughton (1957*a*). A more complete description of work on this and other haemoglobin eactions may be found in the recent review by Gibson (1959).

In using the intermediate-compound hypothesis it is assumed that each haemoglobin solution contains only one reacting species of haemoglobin molecule. The presence of two or more distinct haemoglobins having appreciably different affinities and hence differing in the rate of formation or dissociation of their compounds with ligands would make the analysis of experimental observations in terms of the intermediate-compound hypothesis open to serious objection.

Harris & Warren (1955) have shown that adult sheep haemoglobin is found in two electrophoretically distinct forms. These can occur separately or together in the blood of any individual sheep, and are genetically controlled. Thus three groups of sheep may be distinguished in this respect; Type A sheep possessing only haemoglobin A, the haemoglobin migrating more rapidly towards the anode at pH 8·6, Type B sheep with haemoglobin B and Type AB sheep having both haemoglobins.

In this investigation the two haemoglobins have been compared in the kinetics of some of their reactions with oxygen and carbon monoxide. The conclusions reached by Roughton, Otis & Lyster (1955) and by Gibson & Roughton (1957*a*) are considered in the light of the results.

METHODS

Samples of each haemoglobin have been obtained from the blood of Type A and Type B sheep. No attempt has yet been made to work with haemoglobin separated from the blood of Type AB sheep. All measurements were made at pH 9·1 for the reasons given by Roughton *et al.* (1955).

Electrophoresis. The two haemoglobins were identified by electrophoresis in trays of starch gel prepared by the method described by Smithies (1955); 0·03 M borate buffer, pH 8·6, was used. Haemoglobins of known type were employed as markers on each tray.

Equilibrium studies

Materials. Freshly drawn blood was defibrinated by shaking with glass beads and then laked by the addition of two volumes of distilled water. Cell debris was removed by centrifuging for 15 min at 25,000 *g*. A solution of sodium tetraborate was added to give a final

haemoglobin concentration equivalent to blood diluted 1 in 4 and 1·9 g $Na_2B_4O_7.10H_2O$/100 ml. The solution was used in this form on the day of preparation. If it was necessary to store this solution during the night or longer, it was first deoxygenated by repeated shaking in an evacuated bottle and washing with oxygen-free nitrogen. The oxygen-free solution was then stored under a positive pressure of nitrogen at 2–3° C. until required. In this form sheep haemoglobin is stable for some days with respect to oxygen-reacting properties (Roughton *et al.* 1955).

Determination of K_1. The method described by Roughton *et al.* (1955) was used to make three determinations on the oxygen equilibrium curve of each haemoglobin in the saturation range 1–3 % at 19° C. In this range the approximation $y'/100 = K_1 p/4$, where y' = % saturation and $p = O_2$ pressure (mm Hg), may be used (with the reservation given later) and this was used to give values of K_1 for each haemoglobin.

Determination of L_4 *and* K_4. Measurements on the carbon monoxide equilibrium in the range 99·0–99·5 % saturation at 19° C were made by the method described by Roughton (1954). From two to four determinations were made for each haemoglobin. Further treatment of these results is given in the next section of this paper. There is at present no satisfactory direct method for studying the oxygen equilibrium curve in this saturation range. However, Roughton (1954) had shown that in this saturation range and at pH 9·1 the oxygen and carbon monoxide equilibrium curves are related, and if L_4 is known K_4 may be found from the relationship $L_4 = MK_4$. M is the value of

$$([\mathrm{COHb}]\ \mathrm{pO_2})/([\mathrm{O_2Hb}]\ \mathrm{pCO})$$

for haemoglobin in equilibrium with such partial pressures of oxygen, pO_2, and carbon monoxide, pCO, that the amount of unsaturated haemoglobin is small. Duplicate determinations of M were made on each haemoglobin sample by the method described by Roughton (1954). In using this method it is assumed that carbon monoxide is conserved. The agreement between duplicates which had been equilibrated for times ranging from 2 to 6 hr confirmed that no serious evolution or consumption of this gas was occurring.

Kinetic studies

A rapidly recording stop-flow apparatus similar to that described by Gibson & Roughton (1955) was employed. All measurements were made at room temperature and corrected to 20° C by using the appropriate temperature coefficient.

Determination of k_4. The method described by Gibson & Roughton (1959) was used. 0·4 % Manox sodium hydrosulphite solution in 0·2 M borate buffer, pH 9·1, saturated with carbon monoxide at 1 atmosphere pressure, was mixed with haemoglobin solution, blood 1:80, in the same buffer in equilibrium with 80 mm Hg pressure of air. k_4 may be determined from the slope of the plot of log $[O_2Hb]$ against time.

Determination of l_4. A method based on one described by Gibson & Roughton (1957*b*) was employed. Carbon monoxide haemoglobin solution in equilibrium with a minimal amount of dissolved carbon monoxide was mixed with a solution of nitric oxide in the stop-flow apparatus. The replacement reaction which occurs is slow and it was followed by using a pen recorder instead of the usual oscillograph. l_4 may be determined from the slope of the plot of log [COHb] against time. Controls showed that the rate of photodissociation of carbon monoxide haemoglobin due to the light passing through the observation tube was negligible.

Determination of l_1'. Estimates of this constant were obtained by the method described by Gibson & Roughton (1955). Haemoglobin solutions were deoxygenated by shaking in evacuated tonometers and washing with oxygen-free nitrogen. These solutions were mixed with carbon monoxide solutions and the reaction observed. The usual second-order reaction plot of (log [CO] − log [Hb]) against time is not linear, but from the slope at zero time the value of l_1' can be estimated. Duplicate estimates of this constant were made in this way for each haemoglobin. Initial concentrations were determined by means of the Van Slyke manometric apparatus.

RESULTS

Equilibrium studies

Haemoglobin from four animals of each type was used in the investigation of oxygen equilibrium curves in the range 0–3 % saturation. The values of K_1 determined are shown in Table 1. A measure of the accuracy of each determination, computed from the distance between the experimental points and the best line through them, is also given. The approximation employed gives values of K_1 which are about 15 % too high, as terms in

TABLE 1. Values of K_1 ([mm Hg]$^{-1}$) for two sheep haemoglobins; pH 9·1, 19° C

Haemoglobin A		Haemoglobin B	
K_1	S.E. of estimate	K_1	S.E. of estimate
0·153	±0·003	0·113	±0·011
0·163	±0·008	0·123	±0·001
0·174	±0·004	0·124	±0·018
0·166*	±0·011	0·134	±0·014
0·177*	±0·014	—	—
Mean 0·167		0·1235	

* Separate blood samples from one individual.

p^2 cannot be neglected. No correction has been applied, as both haemoglobins should be affected to a similar extent. Two measurements on haemoglobin from one animal but from blood drawn on two different occasions agree within the experimental error. The results reveal a significant difference between the haemoglobins. Haemoglobin A has an affinity for oxygen which is about 30 % higher than that of haemoglobin B under these conditions.

Measurements on the carbon monoxide equilibrium curve of a similar number of haemoglobin samples in the 99·0–99·5 % saturation range show that haemoglobin A has also a higher affinity than haemoglobin B for this ligand. All the experimental points determined are shown in Fig. 1. The accuracy achieved was not as high as that achieved by Roughton (1954), but it is sufficient to show a definite difference in affinity.

Because of this lower accuracy, the mathematical procedure described by Roughton (1954) for determining the value of L_4 could not be applied to every haemoglobin studied. Typical values of L_4 appear to be 560 (mm Hg)$^{-1}$ for haemoglobin A and 340 (mm Hg)$^{-1}$ for haemoglobin B.

Values of M determined for the same haemoglobin samples are shown in Table 2. The average disagreement between duplicate determinations was less than 2 %. No difference is apparent between the haemoglobins in this respect. Two samples of haemoglobin B from one animal gave values

of M differing by several times the experimental error on different occasions. The cause of this difference is not known. The range of values found was similar to that found by Roughton (1954).

It may be inferred from the value of M and from the results on the carbon monoxide equilibrium that haemoglobin A will have a higher affinity for oxygen than haemoglobin B. Typical values of K_4 are 2·2 and 1·4 (mm Hg)$^{-1}$, respectively.

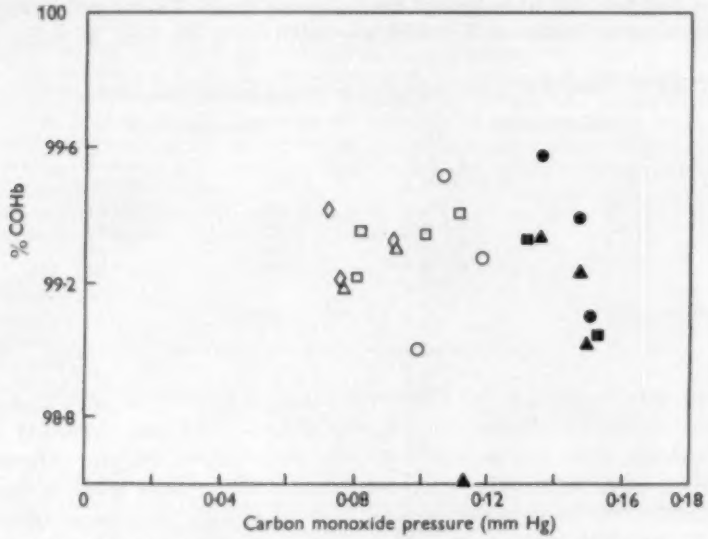

Fig. 1. Determinations on CO equilibrium curves of two sheep haemoglobins; 19° C, pH 9·1. Haemoglobin A △○□◇; haemoglobin B ▲●■.

Table 2. Values of M for two sheep haemoglobins; pH 9·1, 19·0° C

Haemoglobin A	Haemoglobin B
263	260*
260	224*
242	258
252	231
Mean 254·2 ± 12	243·2 ± 19

* Separate blood samples from one individual.

Kinetic studies

Values of k_4 were determined at pH 9·1 and 20° C for six samples of haemoglobin A, three of these coming from one animal, and for five samples of haemoglobin, B, two being from one animal. The values are set out in Table 3. The difference between the haemoglobins is highly

significant and is sufficient to produce the difference in affinity for oxygen already noted. The agreement between the three observations on the haemoglobin from one Type A sheep is satisfactory. The difference between the two values for the Type B animal is rather larger than experimental error.

TABLE 3. Values of k_4 (sec^{-1}) for two sheep haemoglobins; pH 9·1, 20° C

	Haemoglobin A	Haemoglobin B
	38·1*	54·2
	39·1*	55·9*
	39·4*	60·8*
	42·4	57·0
	42·7	57·0
	41·8	
Mean	40·6	57

* Separate blood samples from one individual.

TABLE 4. Values of l_4 (sec^{-1}) for two sheep haemoglobins, pH 9·1, 20° C

	Haemoglobin A		Haemoglobin B	
	l_4	S.D.	l_4	S.D.
	0·0386	±0·0013	0·0459	±0·0018
	0·0341	±0·0012	0·0416	±0·0014
	0·0332	±0·0025	0·0443	±0·0029
	0·0351	±0·0022	0·0455	±0·0011
Mean	0·0355		0·0443	

S.D. of observations about mean.

TABLE 5. Values of l_1' (mM^{-1} sec^{-1}) for two sheep haemoglobins, pH 9·1, 20° C

	Haemoglobin A	Haemoglobin B
	876*	774
	857*	815
	815*	835
	905	859
	824	
	830	
Mean	853	820

* Separate blood samples from one individual.

l_4 was determined for four samples of each type of haemoglobin under the same conditions as k_4. The means of at least five determinations on each sample are shown in Table 4, with estimates of the standard deviation of these determinations about the mean. A difference between the haemoglobins similar to that observed in the case of k_4 may be seen.

Estimates of l_1' at 20° C and pH 9·1 for six samples of haemoglobin A and four samples of haemoglobin B are shown in Table 5. These figures are the means of two determinations for each haemoglobin made with the

same solution of reduced haemoglobin but with different concentrations of carbon monoxide. The average difference regardless of sign between these duplicate determinations was about 8%. The two haemoglobins do not appear to differ significantly in this constant.

DISCUSSION

The results set out in the previous section show that haemoglobin A has an affinity for oxygen in the upper and lower ranges of saturation which is about 30–50% higher than that of haemoglobin B. This is in agreement with the finding of Huisman, van Vliet & Sebens (1958) over the middle range of saturation. It is not certain that the equilibrium curve of one haemoglobin could be made to coincide with that of the other by merely altering the scale of the pressure axis. The difference in affinity may be more pronounced at high saturations than at low.

The dissociation constants appear to be responsible for the major part of this difference in affinities. The values of k_4 observed can explain the differing affinities at high saturation. Evidence on the situation at low saturation has come from some preliminary measurements made, in collaboration with Roughton, on k'_1 for each haemoglobin by the method described by Gibson & Roughton (1958). Similar values of this constant were obtained for each haemoglobin. The finding of different values of K_1 for each haemoglobin must be due to differences in k_1.

The rate of dissociation of ligands from haemoglobin–ligand compounds has been shown by Gibson & Roughton (1957*b*) in many cases to depend on the activation energy of this process. A difference in activation energy of 250 cal would be sufficient to account for the values of k_4 observed. Techniques capable of higher accuracy than those at present available would be necessary to test this experimentally. The interpretation of this and other differences in reactivity of the haem groups in terms of differences in their protein environment must await further knowledge on the structure of these haemoglobins.

The difference in affinity shown by these haemoglobins for oxygen is paralleled by a similar difference in affinity for carbon monoxide. No method has been developed to determine the carbon monoxide equilibrium curve at low saturations but it seems likely that the difference between the haemoglobins in their affinity for this gas demonstrated at high saturations should extend over the whole range of saturation. Again all the available evidence lays the responsibility on the rate constants of the dissociation reactions.

It is possible in some cases to use the results of this investigation to determine the probable nature of haemoglobin samples used in previous

researches before the two types of sheep haemoglobin were discovered. Roughton *et al.* (1955) have published values of K_1, K_2, K_3 and K_4 for four samples of sheep haemoglobin. Comparison of equilibrium curves calculated from these constants with experimental results obtained in this investigation show that three of the four haemoglobin samples they studied were of haemoglobin B, while the fourth (Sheep D), was either a mixture of haemoglobins A and B or haemoglobin A alone. This conclusion is consistent with what is known of the origin of these haemoglobin samples, blood from sheep being slaughtered at Cambridge abattoirs. In the experience of the author, the majority of such animals are Type B. This would be expected from the known breeds of these animals (Border Leicester and Suffolk Down) and from information given by Evans, Harris & Warren (1958) on the distribution of these haemoglobins in British breeds of sheep. The majority of animals in these breeds are Type B. The findings of Roughton *et al.* (1955) must, therefore, escape to a large extent any criticism based on possible non-homogeneity of the haemoglobin samples used.

The same conclusion may be reached concerning the work by Gibson & Roughton (1957*a*) on the combination of carbon monoxide with haemoglobin. Since the haemoglobins react at the same rate it is not possible to determine the nature of the haemoglobin samples studied by these workers, but for the same reason no serious discrepancies should have arisen even if mixed haemoglobin samples were used

Because the difference between the haemoglobins is not large, most other investigations on the kinetics of haemoglobin reactions which have been carried out would have been unaffected in their main conclusions, even if mixed haemoglobin samples had been employed. Nevertheless, for future research it would seem advisable to study haemoglobins of known type.

SUMMARY

1. The two electrophoretically distinct haemoglobins which occur in adult sheep have been compared in some of their reactions with oxygen and carbon monoxide at pH 9·1.

2. Equilibrium measurements show that the haemoglobin which is fast-moving on electrophoresis at pH 8·6, haemoglobin A, has an affinity for oxygen and for carbon monoxide 30–50 % higher than that of haemoglobin B.

3. Measurements on the rates of dissociation of the oxygen and carbon monoxide compounds of each haemoglobin show that the difference in affinities is mainly due to a difference in the dissociation rates of these compounds.

4. This is confirmed by measurements on the combination rate of carbon monoxide with haemoglobin. No significant difference between the haemoglobins appears in this respect.

5. The effect of these findings on the validity of previous kinetic and equilibrium studies is considered.

I am indebted to Dr A. D. Bangham and his technical staff at the A.R.C. Institute of Animal Physiology, Babraham, for providing samples of sheep blood. My thanks are due to the County Armagh Education Committee and the Northern Ireland Ministry of Education for grants. I am especially grateful to Professor F. J. W. Roughton, F.R.S., for valuable advice and encouragement during the conduct of the research described in this paper.

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AN INVESTIGATION OF NUCLEUS GRACILIS OF THE CAT BY ANTIDROMIC STIMULATION

By G. GORDON and W. A. SEED*
From the University Laboratory of Physiology, Oxford

(*Received* 7 *November* 1960)

It can be assumed that the dorsal column nuclei and medial lemniscus provide an orderly system of topographical projections through which information from the whole body surface reaches the thalamus. A particular part of the body is 'represented', throughout the long axis of nucleus gracilis, by a rostral section whose cells have large receptive areas, a middle section with small receptive areas and considerable mutual inhibition, and a caudal section with intermediate receptive areas (Gordon & Paine, 1960). These sections differ functionally; and we felt that an investigation of their projections, in the lemniscus and elsewhere, would help one to understand the meaning of this arrangement. It would also help to show in what form the thalamus receives its information.

Antidromic stimulation is a useful means of studying projections (see, e.g. Lundberg & Oscarsson, 1959), and its analytical value in studying the organization of nucleus cuneatus has been shown by Amassian & de Vito (1957). In this paper we describe antidromic excitation from the medial lemniscus and cerebellum: a brief description of the results was given earlier (Gordon & Seed, 1960).

METHODS

All the experiments were done with cats anaesthetized with sodium pentobarbitone. External records were made from single cells in nucleus gracilis with glass micropipettes, filled with 3 M-KCl and with a resistance at 50 c/s of 3·5–7 MΩ. The techniques for exposing the nucleus, for holding the animals and for inserting the electrodes, have been described in detail in a previous paper (Gordon & Paine, 1960).

Orthodromic stimulation

The size and distribution of the peripheral receptive area of each cell which responded to tactile stimulation of the skin was investigated by moving hairs with a glass rod or touching bare skin (e.g. the pads of the foot) with a camel-hair brush. When accurate timing of a tactile stimulus was needed, the electro-mechanical transducers described by Gordon & Paine (1960) were used.

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In all experiments attempts were made to activate individual cells in nucleus gracilis antidromically by stimulating electrically at sites to which their axons might project (either

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Antidromic stimulation

In all experiments attempts were made to activate individual cells in nucleus gracilis antidromically by stimulating electrically at sites to which their axons might project (either

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the medial lemniscus or the anterior lobe of the cerebellum). Stimuli could be applied singly, in pairs, or in brief high-frequency trains. They were square pulses of 60 μsec duration, isolated from earth by transformer coupling.

Medial lemniscus. Stimuli were applied through buried electrodes. The electrode holder consisted of five parallel stainless-steel tubes, 23 mm long and spaced 1·25 mm apart, clamped between two suitably grooved Perspex plates. The tubes, which were used as guides, had an internal bore just allowing passage of an insulated steel needle of about 0·6 mm diameter. A number of these needles were prepared before each experiment: they were adjusted by attaching 'stops' of solder near the eye, so that when the needles were pushed home into the guides some 28 mm of shaft projected beyond the lower end of the guides and their tips were level. The electrodes used for stimulation were insulated with varnish except for about 1 mm at the tip, the diameter of this terminal millimetre being about 0·15–0·2 mm.

A preliminary exploration was always made with a recording electrode inserted in the centre tube of the electrode holder. This electrode was a steel needle similar to those described above, but insulated up to its tip and more finely tapered: the resistance of such electrodes in Ringer's solution was 20–30 kΩ. The electrode was inserted by a manipulator aligned on Horsley–Clarke co-ordinates into the probable position of the medial lemniscus at the caudal end of the thalamus. Brushing sounds heard in the loudspeaker on tactile stimulation of the contralateral hind limb were a guide to the position of the lemniscus. In frontal planes 4–5 the most satisfactory responses were usually found 5–7 mm from the mid line and 17–20 mm deep to the cortical surface. When this position had been found, the recording electrode was removed, and stimulating electrodes were inserted into the guides of the electrode holder. In early experiments one stimulating electrode was placed on either side of the recording site (electrode separation 2·5 mm). In later experiments five stimulating electrodes were inserted in a transverse row, the middle one through the guide previously occupied by the recording electrode (electrode separation 1·25 mm). Some needles fitted rather loosely in the guides, so that their tips did not always have the equidistant spacing of the guides themselves: this accounts for irregularities in the spacing of the tracks in Text-fig. 3*a*, and also for electrode 3 in this figure not coinciding transversely with the position of the recording electrode. A system was available whereby stimuli could be applied between any two adjacent electrodes, with reversible polarity. The positions of the electrodes were afterwards found histologically.

Cerebellum. In this preliminary investigation the only region explored by stimulation was a small area of the cortex of the anterior lobe. This area corresponded to the region in which Adrian (1943) and Snider & Stowell (1944) found responses to tactile stimulation of the hind limbs.

The occipital pole of the cerebral hemisphere was removed by suction, and the anterior lobe of the cerebellum exposed by nibbling away part of the bony tentorium cerebelli with bone forceps. After opening the cerebellar dura it was usually possible to see the lateral part of the first seven or eight folia of the anterior lobe. The wound was then filled with warm paraffin.

The cortex was stimulated through a pair of silver-ball electrodes about 3 mm apart, placed on the surface of the 6th and 7th folia. The site from which responses could be obtained at lowest threshold was found by making small adjustments of position.

Measurement and notation. The positions of responses in the rostrocaudal dimension of nucleus gracilis are given on a scale of millimetres, zero being the rostral border of the nucleus (see Gordon & Paine, 1960).

RESULTS

All our observations from nucleus gracilis were of single neural units; and we propose, for reasons given before (Gordon & Paine, 1960), to refer

to these responding units as cells. We have confined ourselves here to cells with cutaneous receptive areas wholly or partially upon the hind foot, below the ankle. Nucleus gracilis may be divided, from the point of view of the receptive areas of its cells, into three sections; and we shall refer throughout to the following mean figures for the receptive areas of the cells in the different sections: rostral section (0–4 mm) 58·9 SE. $\pm$ 8·7 cm^2 (n = 115); middle section (4–7 mm) 4·6 $\pm$ 0·98 cm^2 (130); and caudal section (7–12 mm) 15·0 $\pm$ 3·2 cm^2 (94). The difference between these figures and those given by Gordon & Paine (1960) depends only on a different method of expression of the same data.

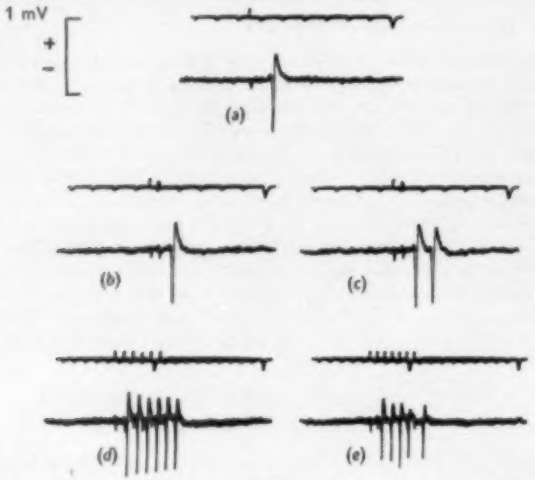
The cutaneous receptive areas of cells in nucleus gracilis are distributed over a very wide range, the ratio between the largest and the smallest being approximately 1:1000. It was convenient to classify these receptive areas into groups with limits successively doubling (0·5–1 cm^2, 1–2 cm^2, 2–4 cm^2, etc.). Areas measured during experiments are necessarily approximate, and such a method of grouping covers the range satisfactorily. The best way of presenting these data would be in the form of 12 histograms, expressing the distribution of receptive areas in each of the 12 1-mm steps along the nucleus. Text-fig. 1 of Gordon & Paine (1960) presents the data in a very compressed form, using mean figures for receptive area in each 1-mm step which are geometric means, giving less weight to individual observations of large receptive area. This figure expresses the general situation conveniently; but arithmetic means would give better values for the mean receptive areas. The figures given above are therefore derived from arithmetic means, using the convention that all areas in the group 0·5–1 cm^2 were 0·75 cm^2, all in the group 1–2 cm^2 were 1·5 cm^2, etc.

Interpretation of responses to lemniscal and cerebellar stimulation

Antidromic excitation. The great majority of responses formed a group with properties which strongly suggested that they resulted from antidromic excitation. In this case a single shock to the lemniscus or cerebellum evoked a response consisting of only one spike (Text-fig. 1*a*). The threshold for this spike was constant; and its latency varied by no more than 3%, without any relation to the strength of the shock. In the population fired in this way by lemniscal stimulation, the latencies were distributed between 0·86 and 2·85 msec, 75% lying between 1 and 2 msec. In the population fired by cerebellar stimulation, latencies lay between 1·0 and 1·9 msec.

The period of unresponsiveness following a spike was tested by pairs of shocks, the second following the first at progressively shorter intervals, with both shocks at twice threshold strength (Text-fig. 1*b* and *c*). The earliest response to a second shock occurred with shock intervals ranging between 0·4 and 0·9 msec in the population studied. In the response to a brief high-frequency train of shocks (Text-fig. 1*d* and *e*) the stimulus frequency was followed accurately, for the first five shocks, up to maxima between 670 and 1320 shocks/sec, with a mean maximum frequency for

the population of about 900/sec. It is unlikely that responses with properties of this kind are mediated synaptically. There is no definite upper limit to the frequency which can be followed in a synaptic system; but the highest we have seen recorded was 640/sec, in a spinal interneurone excited monosynaptically (Eccles, Fatt & Landgren, 1956). All our maxima are higher than this; and Amassian & de Vito (1957) give 380/sec as the maximum frequency followed by comparable cells in nucleus cuneatus in response to synaptic excitation through the dorsal columns.



Text-fig. 1. Antidromic responses of a cell in the rostral section of nucleus gracilis to lemniscal stimulation. Threshold was 0·6 V, and all records were made with twice-threshold shocks. (*a*) response to a single shock, latency 1·0 msec; (*b*) and (*c*) responses to pairs of shocks set at a critical interval giving two responses in approx. 50% of observations, interval 0·4 msec; (*d*) and (*e*) responses to trains of shocks at 1220/sec and 1490/sec respectively. Time markers at 1 and 10 msec intervals appear on the upper trace of each record, projecting downwards: the upward-projecting marks on the same traces signal the stimuli.

Trans-synaptic excitation. A minority of our responses failed to satisfy any of the criteria of antidromic conduction which have just been given, and we have assumed that these responses were mediated synaptically. The responses were of longer latency, falling between 3·3 and 22 msec; and latency usually shortened with increasing strength of shock. The response to a single shock was sometimes repetitive. The period of unresponsiveness after a single shock, measured in the same way as before, was 1·5–4·5 msec; and frequency-following was poor, the highest recorded being 540/sec, with the rest considerably lower than this. Trans-synaptic responses were

seen with lemniscal but not with cerebellar stimulation, and their thresholds were comparable with those of antidromic responses from the lemniscus in the same experiment.

Evidence establishing that responses can be attributed to stimulation of the medial lemniscus

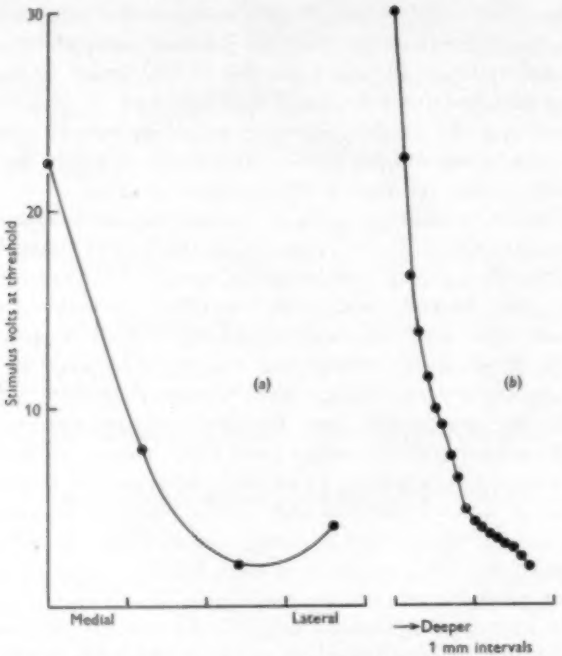
The anatomical complexities of the thalamus and mid-brain make it specially important to identify both the position occupied by stimulating electrodes inserted here and also if possible the particular structures whose stimulation gave rise to the responses that were seen. In this case we were able to show that the points from which responses were elicited at lowest threshold were grouped together in a circumscribed region corresponding closely to the known position of the medial lemniscus.

In experiments conducted specially for this purpose a transverse row of five stimulating electrodes was inserted in the caudal thalamus. It was reasonable to suppose that the lemniscus lay at about the centre of this row and at about the same depth as the tips of the electrodes (see Methods). For each cell which responded antidromically in the contralateral nucleus gracilis, the threshold for stimulation was found between each adjacent pair of electrodes in turn, giving a set of four readings from which a graph was constructed (see Text-fig. 2*a*). Reversal of the polarity of the stimulus allowed another set of four readings to be made, giving another curve with its minimum slightly displaced to one side following the slightly changed distribution of cathodal currents. All the complete curves were strikingly uniform, threshold rising approximately exponentially from its minimum with distance, and being rather more than doubled 1 mm away. A similar relationship probably exists when the spatial co-ordinate is vertical; but in our case such measurements could only be made by moving the electrodes more deeply or superficially through the brain, with some consequent drag on the whole tissue. The curve in Text-fig. 2*b* was obtained by withdrawing the electrodes in measured steps, using the pairs of electrodes giving the lowest threshold in the transverse plane.

The sets of threshold readings from one such experiment, with a transverse row of electrodes, are given in Table 1. The reading representing the lowest measured threshold in each set is marked with an asterisk, and it will be seen that most of these minimum readings are related to electrode 3, and the remainder to electrode 4, as cathode. Plate 1 shows a photomicrograph of a section of the brain of this animal, cut in the plane of the stimulating electrodes; and Text-fig. 3*a* is a tracing of this photograph showing the positions of the electrodes, numbered as they are in Table 1. Text-fig. 3*b* is a figure re-drawn from Ranson & Ingram (1932), showing the position and size of the degenerated medial lemniscus at this level. It

is clear from these figures that the area of tissue which lies between the two minimum-threshold electrodes must include, and is probably identical with, the medial lemniscus.

This localization and grouping of minimal-threshold points was found in all our later experiments in which a transverse grid of electrodes was used,



Text-fig. 2. Graphs showing the change in threshold for the antidromic response of a single cell in nucleus gracilis, occurring with change of stimulus position in the caudal thalamus.

(*a*) Threshold changes with alterations of transverse position. The abscissal positions of the four points give the transverse spacing of the four electrodes used successively as cathodes.

(*b*) Threshold changes for another cell with alteration of vertical position. The electrodes were withdrawn in steps from the lowest-threshold point.

and from these experiments some 35 % of our experimental results were obtained. Histological evidence from almost all the earlier experiments in which responses resulted from stimulating this region showed that the single pair of electrodes embraced the probable position of the lemniscus with much the same precision. In one experiment where the thresholds were of the order of 15–20 V, the electrode tips were afterwards found to

have been about 1 mm too superficial. As we developed experience of this technique and learned to adjust the depth of the electrodes to a position giving uniformly low thresholds, the proportion of cells in nucleus gracilis which could be fired antidromically increased slightly but significantly. It is of some interest that such small adjustments of depth, combined with selection of the appropriate electrodes when a row of these had been inserted, always lowered the threshold for an individual cell to values between 0·6 and 1·0 V with the stimulating pulses and conditions described.

TABLE 1. Thresholds (V) for antidromic excitation of cells in nucleus gracilis by lemniscal stimulation, related to the transverse position of the stimulating cathode

Cell no.	No. of electrode used as cathode					Anode medial (*M*) or lateral (*L*) to cathode
	1	2	3	4	5	
1	—	9·2	3·9*	5·2	33·6	*M*
	24·9	5·5	2·2*	20·0	—	*L*
2	—	14·0	2·1*	2·7	7·2	*M*
	33·9	6·4	1·1*	2·3	—	*L*
3	—	32·9	6·4*	8·9	15·6	*M*
	46·7	24·4	3·2*	8·7	—	*L*
4	—	14·0	4·4	3·0*	4·8	*M*
	—	—	—	—	—	—
5	—	8·2	1·8†	1·8†	—	*M*
	31·4	3·8	0·7	—	—	*L*
6	—	19·3	4·8	2·7†	—	*M*
	45·0	15·0	2·3†	—	—	*L*

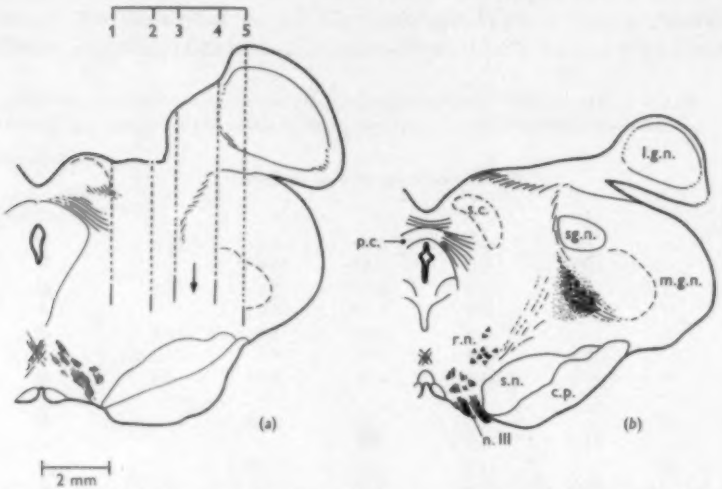
The numbers given to the electrodes (1–5) correspond to those in Text-fig. 3(*a*), which deals with the same experiment. All cells for which such data were obtained in this experiment are included. Incomplete or missing series are due to the loss of the cell through damage. * indicates the lowest-threshold point in series where a minimum was established. † indicates the lowest-threshold point in incomplete series: these values are so low that they must be at or very near the true minimum.

Distribution of antidromic and trans-synaptic responses in nucleus gracilis

We have tried as far as possible to examine the responses of cells over the whole of the rostral 12 mm of nucleus gracilis, within which functional differentiation has been shown by Gordon & Paine (1960). Particular attention was given to antidromic excitation from the contralateral medial lemniscus. The results of this investigation are summarized in Text-fig. 4.

Antidromic lemniscal responses. It was found that cells in all parts of nucleus gracilis could be fired antidromically from the contralateral lemniscus. No such responses were seen in a limited search in the nucleus of the other side (i.e. ipsilateral with respect to the lemniscal electrodes), and it may be assumed that ipsilateral projections are sparse or absent. The great majority of cells in the *middle* section of the nucleus (between 4 and

7 mm from the rostral end) were fired. Of all the cells studied here, 83% were fired (see Text-fig. 4*a*); but with the slightly improved technique of the later experiments this figure increased to 93% (of 45 cells). The percentage from these later experiments is stressed here and elsewhere because of this improved technique. The cells in this part of the nucleus



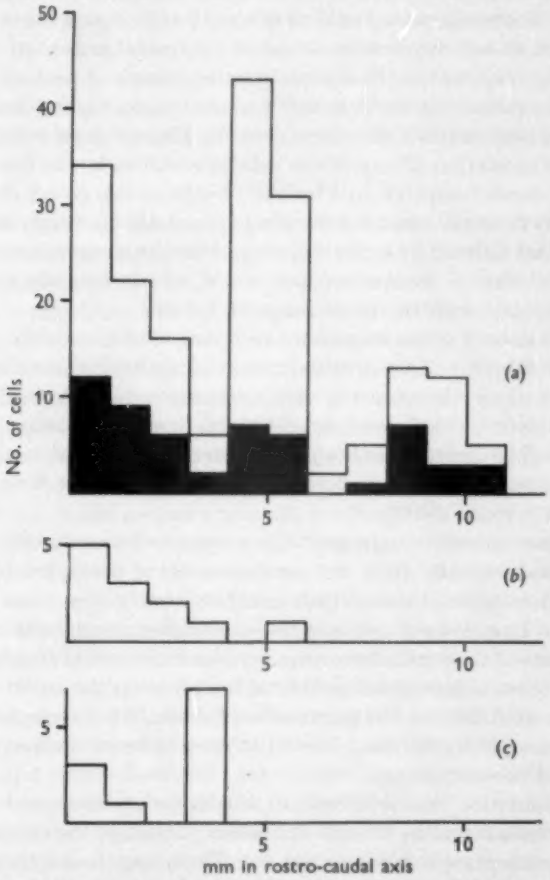
Text-fig. 3. (*a*) Diagrammatic tracing of a transverse section of brain, showing the positions occupied by the stimulating electrodes in the experiment illustrated by Text-fig. 2, Plate I and Table I, and discussed in the text. The electrodes are shown as interrupted lines, with a solid line at the tip of each showing the position and length of the non-insulated part. The tip of the arrow shows the position from which lemniscal responses were recorded in this experiment. Evidence from adjacent serial sections was taken into account in establishing the position of the electrode tips. (*b*) Figure showing the position of the degenerated medial lemniscus (the densely dotted region in the centre of the figure) at about the same antero posterior position as in (*a*). Redrawn from Fig. 1 (Plate 1), Ranson & Ingram (1932), with permission. c.p., cerebral peduncle; l.g.n., lateral geniculate nucleus; m.g.n., medial geniculate nucleus; n. III, oculomotor nerve; p.c., posterior commissure; r.n., red nucleus; s.c., superior colliculus; sg.n., supragenicular nucleus; s.n., substantia nigra.

have uniformly small skin receptive areas (mean 4·5 cm^2), and the mean receptive area for the cells fired antidromically did not differ significantly from this value. Our present evidence shows that almost all of them project rostrally in the contralateral lemniscal system.

In the part of the *caudal* section of the nucleus which was investigated only 60% of cells were fired antidromically (see Text-fig. 4*a*). This figure is derived from a sample of only 36 cells and is clearly not as valuable as

that for the middle section. The receptive area for these cells did not differ significantly from the mean figure of 14·3 cm² for cells in this section.

In the *rostral* 4 mm of the nucleus 63% (of the whole population of 78 cells) were fired antidromically from the lemniscus (see Text-fig. 4*a*). The mean receptive area of these cells did not differ significantly from the mean figure of 51·1 cm² for the rostral section. This part of the nucleus was extensively investigated with the better technique of the later experiments,



Text-fig. 4. Histograms showing the distribution of different classes of cell in 1 mm limits along the rostrocaudal axis of nucleus gracilis. (*a*) Comparison between cells fired antidromically from the medial lemniscus (□) and those not so fired (■). (*b*) Cells fired trans-synaptically from the medial lemniscus. (*c*) Cells fired antidromically from the anterior lobe of the cerebellum.

in which again only 63% (of 41) cells were fired, and we are therefore inclined to accept that a substantial group of these cells is not accessible to this form of stimulation. Presumably these cells either project in paths other than the lemniscus, or have axons ending in the nucleus itself. This conclusion gets some support from the remaining evidence given below.

Trans-synaptic lemniscal responses. Responses to lemniscal stimulation which did not have the characteristics of antidromic excitation (see above) were studied intensively in a small number of experiments. The striking fact about these responses was that of the 12 cells that were satisfactorily investigated all but one were confined to the rostral section of the nucleus (see Text-fig. 4*b*); and in these experiments we were at particular pains to examine the rest of the nucleus under conditions in which these responses would have been found if they were present. There was no evidence of true antidromic excitation of any of the cells in addition to the trans-synaptic effect. The mean receptive area for the 12 cells in this group did not differ significantly from the mean for the whole population in this rostral section. The responses differed from the majority of the population, however, in the rather small size of their action potentials, which were always less than 1 mV, compared with the usual range of 1–3 mV.

We have already given reasons for believing that stimulation was in fact confined to the immediate neighbourhood of the medial lemniscus; and it seems very likely that trans-synaptic responses to such stimulation are brought about by collateral branches of lemniscal axons, within the nucleus, ending directly or through an interneurone on the cell observed. This view has already been expressed by Amassian & de Vito (1957) and by Gordon & Paine (1960).

Antidromic cerebellar responses. Our attempts to excite cells in nucleus gracilis antidromically from the cerebellum were prompted by finding a substantial number of cells which could not be excited from the medial lemniscus. Responses to cutaneous stimulation have been recorded in various parts of the cerebellar cortex. Snider & Stowell (1944) found tactile responses to stimulating the ipsilateral hind foot on the anterior lobe, and from both hind feet on the paramedian lobule. No histological evidence seems to exist, however, for a direct pathway between nucleus gracilis and any part of the cerebellum.

Our stimulation was confined to the anterior lobe; and antidromic responses were found in 11 cells of nucleus gracilis in the small number of these experiments which we carried out. These responses were all recorded from the rostral 4 mm of the nucleus (Text-fig. 4*c*); and we could find no sign of cerebellar antidromic excitation in other parts of the nucleus in spite of intensive search. Ten of these cells were in the nucleus of the same side as the cerebellar stimulus, and one on the opposite side. Their mean

receptive area (110 cm^2) was considerably higher than the mean for the whole population in this section (51 cm^2); but the number of cells involved is too small to establish a significant difference.

It is interesting that three of the cells fired antidromically from the ipsilateral cerebellum were also fired antidromically from the contralateral medial lemniscus. The only conclusion to be drawn from this is that some axons leaving the nucleus send branches to both these destinations.

Cells responding to movement of joints. Gordon & Paine (1960) found that some 10% of cells in nucleus gracilis responded to stimuli such as movement of joints or deep pressure. It may be of interest that, of all such cells found in the present investigation, none was fired antidromically from the contralateral medial lemniscus; but the number of cells was small and a more extensive investigation of this question is still necessary.

DISCUSSION

The main object of this investigation was to find whether all the three functional subdivisions of this nucleus described by Gordon & Paine (1960) project uniformly to the thalamus in the lemniscal system. Our results show clearly that they do not. The almost complete projection from the middle section establishes clearly that the information contained in a large population of cells with small receptive areas is made available to the thalamus. These cells, in common with the cells of other spatially discriminating systems, are subject to a process of mutual inhibition.

In both rostral and caudal sections there were some 40% of cells for which there was no evidence of thalamic projection. The number of cells observed in the caudal section was small and the percentage figure therefore tentative: we have no evidence suggesting the destination of the axons which did not project in the lemniscus. In the rostral section, however, there was definite evidence that some of the cells not projecting in the lemniscus did project to the ipsilateral anterior lobe of the cerebellum.

We have interpreted the trans-synaptic responses to lemniscal stimulation which were seen in the rostral section as the result of excitation of cells by lemniscal axon collaterals. This is a reasonable interpretation in view of our own evidence and that of Amassian & de Vito (1957), and also the fact that Cajal (1909) described such collaterals in lemniscal fibres as they leave the cuneate nucleus. Cajal's account of them is as follows: '(Le cylindre-axe)...émet quelques collatérales destinées aux cellules-sœurs de celle qui lui a donné naissance et se porte en direction antéro-externe;... enfin, il s'unit à d'autres de ses congénères pour entrer dans la voie sensitive ou ruban de Reil médian'. If the cells on which these collaterals ended had projected in the lemniscus we should have seen an early antidromic response succeeded after several milliseconds by the trans-synaptic response.

The fact that this did not happen suggests that these cells either projected by some other path, or that their axons ended in the nucleus itself. Beyond this point any argument about their connexions must be purely tentative, but interesting possibilities exist which deserve further investigation. If these cells were intrinsic (or 'internuncial') cells, and had excitatory endings on other cells in the nucleus, we might expect to record from some of the latter, which if they projected outside the nucleus should show both antidromic and trans-synaptic firing. Amassian & de Vito (1957) describe this dual response in cells of nucleus cuneatus, and one might equally expect to see it in nucleus gracilis. Such a system of excitatory intrinsic cells might provide the basis for the extensive convergence which is needed to explain the large receptive areas of many of the cells in the rostral section. The possibility remains that some proportion of cells fired trans-synaptically are inhibitory in function, isolated examples of inhibition of cells in nucleus gracilis by lemniscal stimulation having been described (Gordon & Paine, 1960).

SUMMARY

1. Cells in nucleus gracilis which responded to tactile stimulation of the hind foot were investigated by giving electrical stimuli to the contralateral medial lemniscus and to the ipsilateral side of the anterior lobe of the cerebellum. The technique for lemniscal stimulation and its anatomical control are described in some detail.

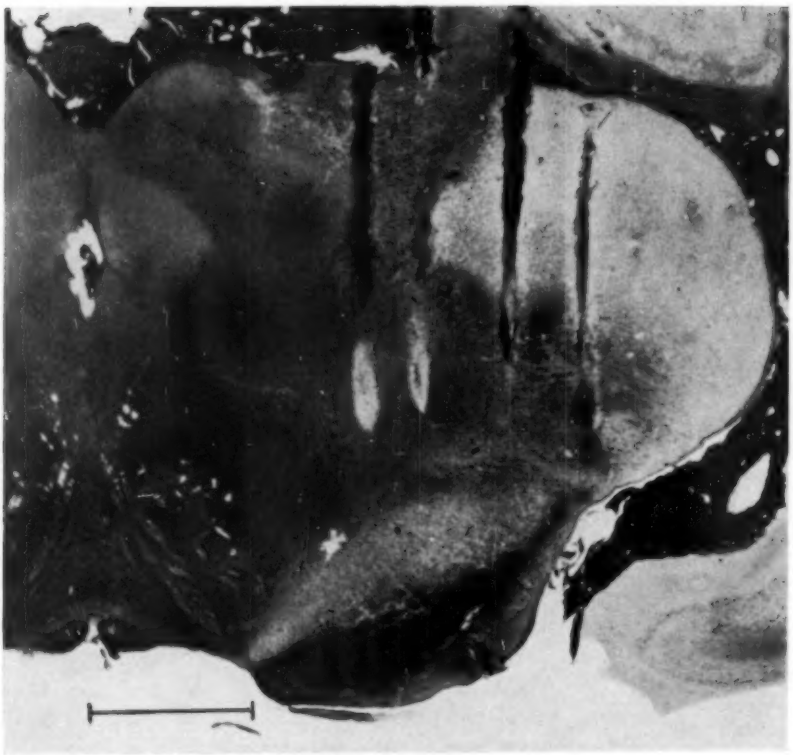
2. Cells throughout the rostrocaudal axis of the nucleus were fired antidromically from the lemniscus. A high proportion of cells were fired in the middle section, where receptive areas are small (93 % of 45 cells in the best series of experiments, and 83 % of the total cells). 60 % (of 36) cells were fired in the caudal section. In the rostral section, where receptive areas are large, 63 % of 41 cells were fired in the best series, and 63 % also of the total of 78 cells.

3. Some cells in the rostral section (but not elsewhere) were fired antidromically from the anterior lobe of the cerebellum.

4. Some cells, almost all in the rostral section, were fired trans-synaptically from the lemniscus, and the significance of these responses is discussed.

5. It was concluded that almost all the cells in the middle section project in the contralateral lemniscus; but that a significant number in the rostral section either project elsewhere (to the cerebellum, for example), or have axons ending within the nucleus (intrinsic cells). It was not possible to reach such definite conclusions about the caudal section, where the sample was rather small.

We should like to thank Mr E. H. Leach for the photomicrograph in Plate 1, and Miss Christine Court for the drawings in Fig. 3.



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EXPLANATION OF PLATE

PLATE 1

Photomicrograph of the transverse section of brain from which Text-fig. 3(*a*) was traced. Four of the electrode tracks can be seen clearly. The most superficial part of the remaining one (the most medial, No. 1 of Text-fig. 3(*a*)) can just be seen: the deeper parts of this, and the track made by the recording electrode, were seen in adjacent sections. 50 μ: Heidenhain's iron-haematoxylin. Scale = 2 mm.

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THE CONTRIBUTION OF THE BRONCHIAL CIRCULATION TO THE VENOUS ADMIXTURE IN PULMONARY VENOUS BLOOD

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There is general agreement that the oxygen tension (pO_2) of the arterial blood is lower than that of the alveolar air; the difference is 5–10 mm Hg in man and 15–25 mm Hg in the dog (e.g. Haab, Piiper & Rahn, 1960). There are three factors which could be responsible for this difference: (1) a resistance to the diffusion of oxygen across the alveolar membrane, (2) uneven distribution of alveolar ventilation in relation to pulmonary blood flow in various parts of the lungs, and (3) the admixture of venous blood to arterialized blood which has left the pulmonary capillaries. With regard to the last factor, one potential source of venous blood is the bronchial circulation which drains blood into the pulmonary circulation via communicating channels between the two vascular beds.

The bronchial arteries arise from the aortic arch and thoracic aorta and supply oxygenated blood to such structures in the lungs as the bronchial tree as far as the respiratory bronchioles, the pulmonary nerves and nerve ganglia, the lymph nodes and the interstitial lung substance (see Miller, 1947). In addition, they may also supply the alveoli in the proximal parts of the respiratory bronchioles, which project between the mesh of anastomosing bundles of bronchial muscle (Le Fort, 1858; Küttner, 1878; Baltisberger, 1921; Miller, 1921). The blood from these structures may take one of two courses. Blood from the bronchial tree as far as the second-order bronchi drains by the bronchial veins into the right atrium, whereas blood from the remaining parts of the bronchial tree as far as the respiratory bronchioles reaches the left atrium via the bronchopulmonary veins, which join the pulmonary veins (Le Fort, 1858; Zuckerkandl, 1881; Berry, Brailsford & I. de B. Daly, 1931; Berry, 1934). These bronchopulmonary veins serve therefore as anastomotic channels between the bronchial and pulmonary vascular systems. A second group of such channels exists in the region of the respiratory bronchioles where the capillary beds of the two circulations anastomose (Küttner, 1878; Miller, 1925). Morphological evidence suggests that in the dog these are the only groups of intrapulmonary anastomotic channels between the two circulations. In man, on the other hand, anastomoses exist at a pre-capillary level as well (Küttner, 1878; Zuckerkandl, 1883; von Hayek, 1940, 1942; Verloop, 1948; Tobin, 1952).

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The view has been held that venous admixture is due, at least in part, to venous blood from the bronchial circulation entering the pulmonary veins. Such a view implies that the venous blood from the bronchial circulation does not take up oxygen from the alveolar air before entering the pulmonary veins and that the functional communicating channels between the two circulations must, therefore, lie largely at a post-capillary level. It is somewhat surprising, however, that the venous nature of the bronchial blood has not hitherto been demonstrated.

The present experiments were undertaken in an attempt to throw new light on this problem. In the first part of this paper evidence is presented which shows that in the dog the bronchial blood becomes venous in its passage through the lungs. Experiments have also been made to discover the site at which the bronchial venous blood joins the pulmonary circulation; these experiments are described in the second part of the paper. Some of our results have already been reported briefly elsewhere (Daly, Aviado & Lee, 1953).

METHODS

All the experiments were performed on dogs anaesthetized with morphine hydrochloride (2 mg/kg subcutaneously) and chloralose (70 mg/kg intravenously). A cannula was inserted in the trachea and the lungs were ventilated artificially by means of a Starling 'Ideal' pump. Manuronate (Wyeth, 50 mg/kg) or heparin (Abbott Laboratories, Ltd., 10 mg/kg) was used to render the blood incoagulable.

Bronchopulmonary blood-flow measurements in the entire dog. After opening the chest in the left fifth intercostal space, the pulmonary artery and vein to the left diaphragmatic lobe were ligated and plastic catheters inserted into their distal ends (Fig. 1). Sometimes the pulmonary vein was too short to cannulate conveniently, and in this case a catheter was put into each of its branches. The outflow ends of the catheters were held at the same horizontal level as the inserted ends. The outflowing blood from the two catheters was collected either in graduated cylinders and then returned via a cannulated femoral vein, or in syringes for analysis. In a few experiments the chest was subsequently closed, the pneumothorax reduced and spontaneous respiration restored. Systemic blood pressure was measured from a femoral artery by means of a mercury manometer.

Separate perfusions of the bronchial and pulmonary vascular systems. The right lung of the experimental dog was isolated and perfused independently through the cannulated right bronchial artery and right pulmonary artery from a donor dog. After bleeding the experimental dog to death, the following structures were ligated: left pulmonary artery and veins, left bronchus, superior and inferior venae cavae, azygos vein, the descending aorta immediately above the diaphragm, the ascending aorta, the left subclavian and brachiocephalic arteries, and the oesophagus above the diaphragm and at the level of the aortic arch. A tape was tied tightly round the heart at the level of the atrioventricular groove.

The details of the perfusion system are shown diagrammatically in Fig. 2. By means of a Dale–Schuster pump (P_1), the right lung was perfused through the right pulmonary artery at a constant blood volume inflow with venous blood from the right atrium of a donor dog. The right bronchial artery was perfused by a second pump (P_2) at a constant pressure with arterial blood from a femoral artery of the donor animal. The connexions were such that the composition of the blood perfusing the bronchial circulation could be changed at will from oxygenated to venous by removing the clamp at X_1 on the tube connecting the inflow sides of the pumps P_1 and P_2 and applying it at X_2 (Fig. 2).

Cannulae were inserted into the left and right auricular appendages and the blood collected from each atrium was measured by means of graduated reservoirs (*a* and *b*) and a stopwatch. The left and right atrial pressures remained constant during the flow measurements. The blood was returned from reservoir (R_1) to the donor dog by a third pump (P_3). Bronchial arterial and pulmonary arterial pressures were measured by means of a mercury and a saline manometer respectively (m_1, m_2).

The procedure adopted for making the isolated right lung preparation was such that perfusion of the pulmonary and bronchial circulations was started within 12 min of the heart stopping. This was achieved by passing threads round all the structures mentioned above and cannulating the left and right atria before bleeding the animal to death.

Blood samples for subsequent analysis were drawn from the bronchial and pulmonary inflow tubes and from the left atrium.

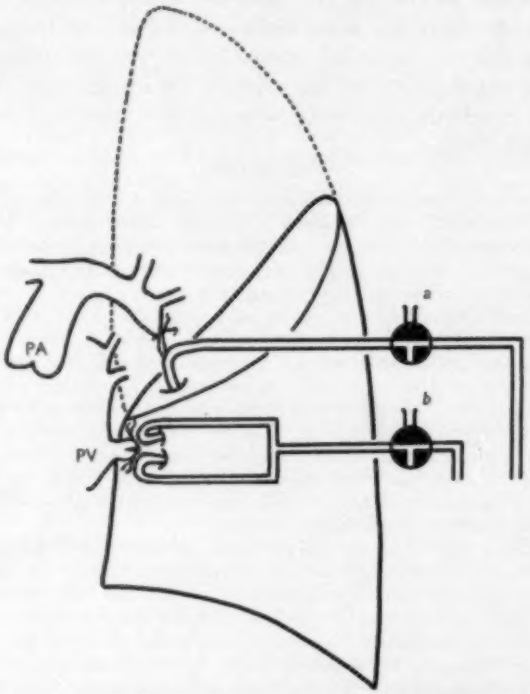

Fig. 1. Diagram showing the method for measurement of bronchopulmonary blood flow. The pulmonary artery and vein to the left diaphragmatic lobe are ligated and catheters are inserted into their distal ends. Blood flow is measured in graduated tubes. The remaining lobes of the left lung and the whole of the right lung (not shown) are normal. Syringes are attached to the three-way taps at *a* and *b*, and after turning the taps through 180° simultaneous blood samples are drawn from the ligated pulmonary artery and vein at a rate slightly slower than the rate of blood flow, thereby maintaining the vascular pressures in the lung lobe constant.

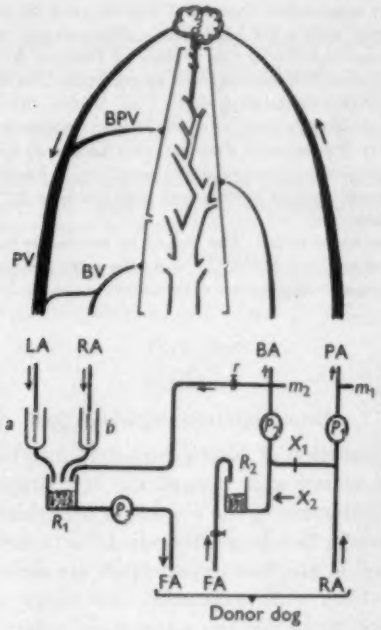

Fig. 2. Diagram showing the method for perfusion of the isolated right lung through the pulmonary artery (PA) and bronchial artery (BA) simultaneously. The upper part of the diagram shows the bronchial tree, the pulmonary artery, pulmonary capillaries and pulmonary vein (PV), the bronchial artery supplying the bronchial tree as far as the respiratory bronchioles, the venous drainage of the bronchial tree via the true bronchial veins (BV) and bronchopulmonary veins (BPV), and the capillary anastomosis between the pulmonary and bronchial vascular systems. The lower part of the diagram shows the arrangement for perfusion of the lung. Venous blood from the right atrium (RA) of the donor dog is perfused through the pulmonary artery of the isolated right lung of the recipient animal by means of pump P_1. Blood from the left and right atria of the isolated lung is returned to the reservoir R_1 via two graduated tubes *a* and *b*, and thence back to a femoral artery of the donor dog by pump P_3. The bronchial circulation is perfused by pump P_2 with oxygenated blood obtained from a small reservoir R_2 which is supplied from a femoral artery of the donor dog. The bronchial arterial pressure is controlled by the resistance *r*, the excess of blood passing through it being returned to the reservoir R_1. The connecting tube joining the input sides of the pumps P_1 and P_2 is normally clamped at X_1. The clamp is removed and applied at X_2 for perfusion of the bronchial circulation with venous blood instead of oxygenated blood. m_1 and m_2 are manometers measuring pulmonary and bronchial arterial pressures respectively. For details see text.

Blood analyses. The samples of blood were analysed in duplicate for oxygen and carbon dioxide contents by the manometric method of Van Slyke & Neill (1924). The values for duplicate samples agreed within 0·2 ml./100 ml. Haemoglobin was determined photometrically as cyanmethaemoglobin by the method of Drabkin & Austin (1935). Oxygen capacity was calculated from the haemoglobin concentration, on the assumption that 1 g haemoglobin combines with 1·34 ml. oxygen.

The oxygen and carbon dioxide tensions of the blood were determined by the microtonometric technique of Riley, Proemmel & Franke (1945) using a 10 ml. Roughton–Scholander syringe. The technical procedures were modified according to Lambertsen, Bunce, Drabkin & Schmidt (1952). Determinations in duplicate were made at 37° C, within 2 hr of withdrawal of the blood specimens.

Determination of lung tissue weight. The weight of wet tissue in the left diaphragmatic lung lobe was calculated as the difference between the total weight of the excised lobe and the weight of blood contained in the lobe, determined by the method of Aviado & Schmidt (1952).

RESULTS

Bronchopulmonary blood flow

Entire dog. The insertion of plastic catheters into the distal ends of the ligated pulmonary artery and vein to the left diaphragmatic lung lobe allowed direct measurement of the volume of bronchial blood draining into the pulmonary vascular bed, hereafter called the bronchopulmonary blood. The results obtained in nineteen experiments are shown in Table 1. In six experiments (nos. 1–6), with open chest and under artificial respiration, the combined blood flows for the pulmonary artery and vein averaged 4·4 ml./min (range 2·1–7·7 ml./min or 0·11–0·36 ml./min/kg body weight). There was no appreciable difference between the blood-flow values for these experiments and those obtained in three others in which the chest was closed and spontaneous respiration restored (Table 1, Expts. nos. 17–19). It was found that, if one of the two catheters was clamped, the blood flow through the remaining catheter increased, the final value being equal to the combined flows when both catheters were patent. In a further ten experiments (Table 1, Expts. nos. 7–16), the bronchopulmonary blood flow was measured in either the pulmonary artery or vein, the vessel not cannulated being ligated. The observed values for blood flow were similar to the combined pulmonary artery and vein flows obtained in Expts. nos. 1–6 (Table 1). The mean blood flow for the nineteen experiments is 4·8 ml./min. These results indicate that, in the lung lobe without its normal pulmonary blood flow, the total measured bronchopulmonary blood flow is the same whether it is collected from either the pulmonary artery, the pulmonary vein or both. It appears that the bronchopulmonary blood collected from one of the two pulmonary vessels can readily use the normal channels between them, presumably the pulmonary capillaries.

It was found that raising the ends of the catheters up to 10 cm above

the level of the pulmonary artery had no appreciable effect on the volume of blood flow.

With regard to the partition of the bronchopulmonary blood flow between pulmonary artery and vein in the *absence* of a normal pulmonary circulation, it will be noted in Table 1 that, with the exception of one experiment (no. 6), about 60% of the combined flow was collected from

TABLE 1. Values for bronchopulmonary blood flow obtained from the distal ends of the ligated pulmonary artery and vein for the left diaphragmatic lobe. PA = pulmonary artery; PV = pulmonary vein; L = ligated

Expt. no.	Dog wt. (kg)	Systemic B.P. (mm Hg)	Blood flow				Proportion of total flow (%)	
			PA (ml./min)	PV (ml./min)	PA+PV (ml./min)	PA+PV (ml./min/kg)	PA	PV
			Open chest					
1	12·5	130	1·6	1·9	3·5	0·28	46	54
2	13·0	130	0·6	2·1	2·7	0·21	22	78
3	21·4	100	2·6	5·1	7·7	0·36	34	66
4	19·1	100	0·5	1·6	2·1	0·11	24	76
5	18·4	150	1·2	4·0	5·2	0·28	23	77
6	13·9	150	3·1	1·8	4·9	0·35	63	37
7	24·8	130	L	7·4	7·4	0·30	—	—
8	16·2	125	4·6	L	4·6	0·28	—	—
9	16·8	110	2·8	L	2·8	0·17	—	—
10	15·9	115	4·0	L	4·0	0·25	—	—
11	13·4	115	2·4	L	2·4	0·18	—	—
12	12·8	117	6·4	L	6·4	0·50	—	—
13	13·2	—	8·5	L	8·5	0·64	—	—
14	12·3	—	3·0	L	3·0	0·24	—	—
15	16·3	135	5·4	L	5·4	0·33	—	—
16	19·1	117	5·2	L	5·2	0·27	—	—
			Closed chest					
17	16·1	90	1·5	2·6	4·1	0·25	37	63
18	18·6	120	3·5	4·7	8·2	0·43	43	57
19	18·4	140	1·2	2·5	3·7	0·20	32	68

the pulmonary vein. The exact reason for this is not known, but presumably the resistance to flow offered by the pulmonary venules and veins is less than the arterioles and arteries, and this may be due to the closer proximity of the bronchopulmonary veins to the pulmonary venous catheter.

Perfused right lung preparation. Four experiments were carried out in which the pulmonary and bronchial circulations were simultaneously perfused. The bronchial arterial pressure varied in different experiments from 80 to 210 mm Hg and the pulmonary arterial pressure from 15 to 24 cm saline. Blood flow from the left atrium was 86–306 ml./min and from the right atrium, 5·7–33·4 ml./min. Because the flow of blood through the pulmonary and bronchial vascular systems was under independent control, it was possible to elucidate their respective contributions to the volume of blood flow to the left and right atria. The contribution of the

bronchial arteries was determined by taking measurements of the left and right atrial outflows before, during and after interruption of flow through the bronchial circulation, produced by lowering the bronchial arterial pressure to zero, pulmonary arterial perfusion being continued throughout the test. The values for outflow during the second period with pulmonary perfusion only represents the contribution from the pulmonary artery. The results of eighteen tests in four experiments are shown in Table 2.

TABLE 2. Values for the distribution of bronchial arterial blood to the left and right atria in the isolated right lung preparation perfused simultaneously through the pulmonary artery with mixed venous blood and through the bronchial artery with oxygenated blood. PA = pulmonary artery; BA = bronchial artery

		Left atrial blood flow			Right atrial blood flow (ml./min)		Distribution of bronchial arterial blood (%)	
Expt. no.	Dog wt. (kg)	From PA (ml./min)	From BA (ml./min)	Contribution from BA (%)	From PA	From BA	To left atrium	To right atrium
22*a*	11·4	138	12	8	1·2	4·5	73	27
b		129	9	6·5	2·7	5·9	60	40
c		180	25	12	5·2	5·7	82	18
d		190	35	15·5	2·0	10·9	76	24
26*a*	13·9	160	0	0	6·1	16·2	0	100
b		159	5	3	10·2	20·9	19	81
c		159	3	2	13·0	20·4	13	87
d		160	7	4	0	32·0	18	82
e		80	6	7	—	—	—	—
27*a*	12·3	147	5	3	—	—	—	—
b		150	5	3	—	—	—	—
c		150	7	4·5	—	—	—	—
d		118	9	7	—	—	—	—
e		103	11	10	1·0	6·8	62	38
f		101	11	10	1·0	6·1	64	36
g		100	5	5	3·0	4·6	52	48
h		96	7	7	7·6	0	100	0
28	13·0	300	6	2	—	—	—	—

The volume of bronchial blood to the right lung only reaching the left atrium averaged 20·3, 4·2, 7·5 and 6·0 ml./min, in the four experiments respectively. This is equivalent to 10·5, 3·2, 6·2 and 2·0 % of the total left atrial blood outflow.

Two other points of interest may be noted from Table 2. First, a small proportion of the pulmonary arterial blood finds its way to the right atrium. A similar finding has been reported previously by I. de B. Daly & von Euler (1932). Secondly, in three experiments (nos. 22, 26 and 27) in which values are available for the distribution of the bronchial *arterial* blood, it was found that 72, 13 and 70 % of the blood respectively drained into the left atrium, the remainder into the right atrium. These values are subject to considerable variation because not all the bronchial arterial blood passes through the lungs; in the present experiments some blood

undoubtedly passed through adjacent mediastinal structures, such as the oesophagus, and then found its way to the right atrium (Miller, 1947). This explanation may account for the comparatively high proportion of the bronchial blood reaching the right atrium in Expt. no. 26.

Gaseous composition of bronchopulmonary blood

The evidence obtained from experiments in which the bronchopulmonary blood flow was measured in the left diaphragmatic lobe of the entire animal suggested that in the absence of the normal pulmonary

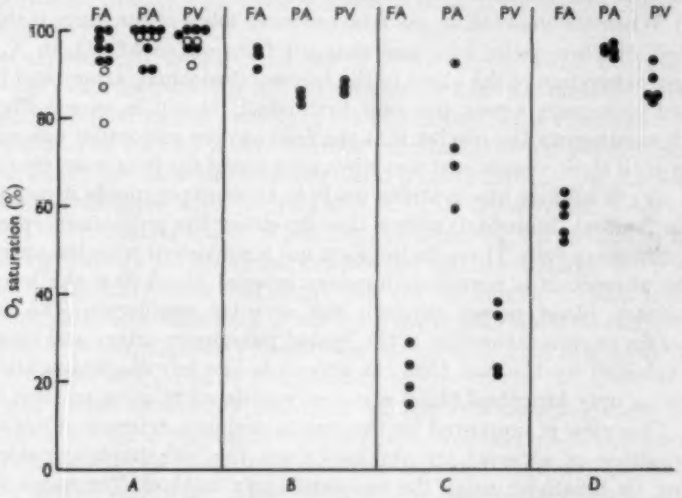

Fig. 3. Oxygen saturation of the blood obtained simultaneously from the femoral (bronchial) artery (FA), and the distal ends of the ligated pulmonary artery (PA) and pulmonary vein (PV) to the left diaphragmatic lobe in dogs with open chest under artificial respiration (●) and with closed chest breathing spontaneously (○). *A*, ventilation of both lungs with air; *B*, ventilation of left diaphragmatic lobe with 5% O_2 in N_2, the remaining lobes of the lungs with air; *C*, ventilation of both lungs with 5% O_2 in N_2; *D*, systemic hypoxia produced by occlusion of the left upper and middle lobe bronchi, the remaining lobes of the lungs being ventilated with air.

arterial blood flow, the bronchial blood passed through the pulmonary capillaries before entering the collecting catheters in the lobar pulmonary artery and vein. Further experimental evidence consistent with this view was obtained as follows.

(1) The oxygen saturation of the blood obtained from the distal end of the ligated pulmonary artery and vein was almost identical with that in the femoral (bronchial) artery (Fig. 3*A*).

(2) When the ventilating gas mixture to the left diaphragmatic lobe was changed from room air to 5% O_2 in N_2, ventilation of the remaining lobes of the left lung and the whole of the right lung being continued with room air, the oxygen saturation of the blood leaving the ligated pulmonary artery and vein diminished in three experiments, in spite of the femoral (bronchial) arterial blood oxygen saturation being maintained above 90%. The final steady-state values are shown in Fig. 3*B*. In these experiments the low-oxygen gas mixture was administered via a bronchospirometric tube inserted into the bronchus to the left diaphragmatic lobe. The position of the tube was checked post mortem.

(3) When the ventilating gas mixture to all lobes of the lungs including the left diaphragmatic lobe was changed from air to 5% O_2 in N_2, the oxygen saturation of the blood in the femoral (bronchial) artery and in the ligated pulmonary artery and vein diminished. It will be seen in Fig. 3*C*, which summarizes the results, that the final oxygen saturation was not the same in all three vessels as it was when all lobes of the lung were ventilated with air; in all four observations made in three experiments it was lower in the femoral (bronchial) artery than in either the pulmonary artery or the pulmonary vein. These findings are not inconsistent with the view that in the absence of a normal pulmonary arterial blood flow the bronchopulmonary blood passes through the alveolar capillaries. The higher values for oxygen saturation in the ligated pulmonary artery and vein may be explained by the fact that the alveoli in the left diaphragmatic lobe receiving only bronchial blood are over-ventilated relative to their blood flow. This view is supported by the results of direct determinations of the composition of alveolar air obtained from the left diaphragmatic lobe during air breathing using the end-expiratory method. The mean values for five observations in three experiments were as follows: pO_2, 142 mm Hg (range 141–145 mm Hg), pCO_2, 8 mm Hg (range 6–10 mm Hg).

It will also be noted in Fig. 3*C* that the oxygen saturation of the pulmonary arterial blood was consistently higher than that in the pulmonary vein. One possible explanation for this is that a higher proportion of the blood obtained from the pulmonary artery compared to the vein passes through the capillary anastomosis and is exposed to the alveolar air, some of the blood collected from the pulmonary vein taking a course via the bronchopulmonary veins. Essentially similar results were obtained in experiments in which systemic hypoxia was produced in dogs ventilated with air by occlusion of the upper and middle lobe bronchi on the left side (Fig. 3*D*).

By contrast, it is of interest that no appreciable difference between the oxygen contents of the pulmonary arterial and venous blood was observed during air breathing. This is probably due to the fact that the oxygen

saturation of the blood lay on the flat part of the oxygen-dissociation curve.

Whereas these observations strongly suggest that, in the absence of a normal pulmonary arterial blood flow at least part of the bronchopulmonary blood passes through the pulmonary capillaries, they provide no evidence as to the composition of the bronchial blood *before* it enters these capillaries, in which it may undergo changes according to the tensions of oxygen and carbon dioxide prevailing in the alveoli. The aim of the experiments now to be described was to obtain information on the gaseous composition of bronchopulmonary blood unaltered by equilibration with alveolar air.

The experiments were carried out on entire dogs in which the bronchopulmonary blood flow to the left diaphragmatic lobe was measured. Ventilation of this lobe was suspended in the expiratory phase of respiration by inflating a small balloon in its bronchus. Exchange of oxygen and carbon dioxide in the alveoli with bronchial blood passing through the pulmonary capillaries continues until the pO_2 and pCO_2 of the alveolar gases equal those of the bronchial blood. When this has occurred, the bronchial blood on passing through the pulmonary capillaries will not undergo any further change, provided the flow and composition of the bronchial blood remains constant, and the metabolism of the lung tissue undergoes no change. In practice, bronchopulmonary blood was sampled at intervals until there was no further change in its composition.

It was found that after occlusion of the left diaphragmatic lobar bronchus there was a gradual reduction in the oxygen content and increase in the carbon dioxide content of the blood sampled from the ligated pulmonary artery and vein. After 60–90 min a steady state was reached. Thus, whereas immediately before occlusion of the bronchus there was little difference between the oxygen content of the femoral (bronchial) arterial blood on the one hand and bronchopulmonary (pulmonary artery or vein) blood on the other (Fig. 3*A*), an appreciable difference developed after 60–90 min occlusion of the lobar bronchus. The final values for oxygen and carbon dioxide contents of femoral (bronchial) arterial and corresponding bronchopulmonary blood are shown in Fig. 4 and are based on fourteen observations from seven experiments. The mean values for the oxygen content of the bronchial arterial and bronchopulmonary blood was 15·3 ml./100 ml. (range 9·3–22·4) and 9·6 ml./100 ml. (range 2·5–20·4), corresponding to oxygen saturations of 86·6% (range 75–99) and 50·2% (range 19–83) respectively. The mean oxygen and carbon dioxide differences between bronchial arterial blood and bronchopulmonary blood was 5·7 ml./100 ml. (range 2·0–8·9) and 2·8 ml./100 ml. (range 0·2–6·7) respectively.

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If, after a period of 60–90 min of occlusion of the bronchus, ventilation of the diaphragmatic lobe was re-established, an immediate increase in the oxygen content and saturation of the bronchopulmonary blood took place. This was evident from the sudden change in colour of the blood in the catheters in the ligated pulmonary artery and vein, and from an analysis of the bronchopulmonary blood.

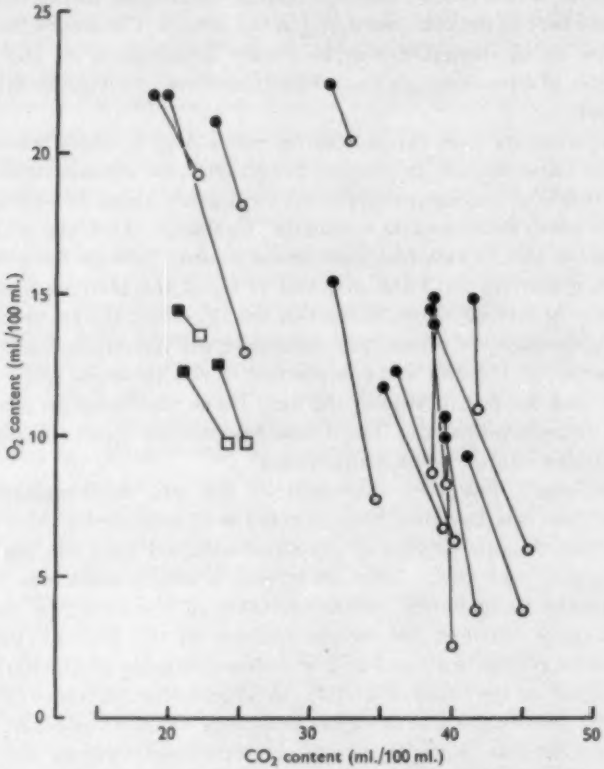

Fig. 4. The values for oxygen and carbon dioxide contents of bronchial arterial blood (●) and of bronchopulmonary blood (○) in seven experiments in which the left diaphragmatic lobar bronchus had been occluded for 60–90 min. The corresponding values obtained in two isolated right lung preparations perfused through the bronchial artery only are also shown (■—□).

These findings were confirmed in two experiments using the isolated right lung preparation perfused through the bronchial circulation only. After ventilation had been stopped for about 90 min, blood samples were drawn from the bronchial arterial tubing and from the left atrium. The

results of three observations are shown in Fig. 4. The mean values for the oxygen content of the bronchial arterial and left atrial blood was 13·2 ml./100 ml. (range 12·3–14·4) and 11·0 ml./100 ml. (range 9·7–13·5), corresponding to mean oxygen saturations of 96·7 % (range 96–97) and 79·7 % (range 74–90) respectively.

These results indicate that the bronchial arterial blood becomes venous in nature on its passage through the pulmonary tissue. In the ventilated lung lobe without its normal pulmonary circulation, however, the bronchial venous blood becomes arterialized before collection in the catheters, through exposure to alveolar air containing a high pO_2 and a low pCO_2.

Functional anastomoses between the bronchial and pulmonary circulations

Our finding that in the absence of the normal pulmonary circulation the bronchial venous blood passes through the pulmonary capillaries raises the question as to whether, under normal conditions of circulation through the lungs, the bronchial venous blood reaches the left atrium by the same route or alternatively joins the pulmonary circulation by way of the bronchopulmonary veins without traversing the pulmonary capillaries. The importance of establishing this point lies in the fact that the admixture of bronchial venous blood will occur only if the bronchial blood joins the pulmonary circulation at a post-capillary level. Two types of experiment were therefore made on the isolated right lung preparation, perfused simultaneously through the pulmonary and bronchial arteries, with a view to gaining information on this point.

(1) *Temporary interruption of bronchial blood flow*

A. Perfusion of bronchial circulation with oxygenated blood. In four experiments the lung was ventilated with room air while perfusion of the pulmonary circulation was carried out with venous blood and of the bronchial circulation with oxygenated blood. It was found that temporary interruption of the bronchial circulation by reducing the bronchial arterial pressure to zero had no consistent effect on the oxygen and carbon dioxide contents of the left atrial blood (nine observations) (Fig. 5*A*). It is possible, however, that the size of the changes in left atrial blood oxygen content may be so small as to lie within the error of the method used. Two series of experiments were therefore carried out with a view to magnifying any changes in composition of the left atrial blood which might take place when the bronchial arterial pressure was reduced to zero.

B. Perfusion of bronchial circulation with venous blood. With the same preparation, with the exception that the bronchial circulation was perfused

with venous blood from the donor dog (Fig. 2) instead of with oxygenated blood, it was found in three experiments that reducing the bronchial arterial pressure to zero caused an increase in oxygen content and oxygen saturation of the left atrial blood in three out of six tests; in the remaining three tests there was no change within the limits of error of the method (Fig. 5*B*). In two of these three tests which showed no alteration in oxygen saturation, values are available for the changes in oxygen tension determined by the direct microtonometric method. It was found that the pO_2 of the left atrial blood increased by 11 and 6 mm Hg respectively. These results therefore suggest that, in the presence of a normal pulmonary circulation, most if not all the bronchopulmonary venous blood enters the pulmonary circulation at a post-capillary level and without traversing the pulmonary capillaries.

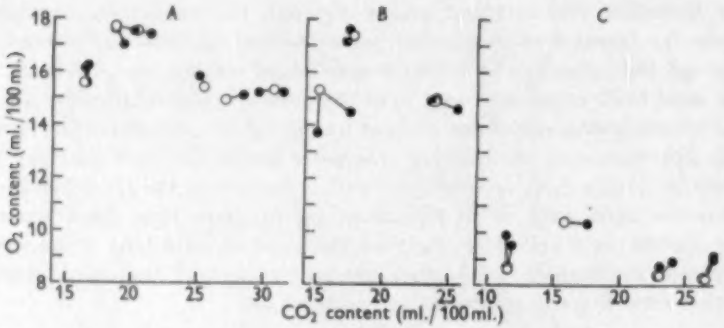

Fig. 5. The oxygen and carbon dioxide contents of the left atrial blood before (●) and during (○) temporary interruption of the bronchial circulation, by reducing the bronchial arterial pressure to zero, in the isolated perfused right lung preparation. Perfusion of the pulmonary circulation with venous blood throughout. *A*, oxygenated blood perfusion of the bronchial circulation; ventilation with room air. *B*, venous blood perfusion of the bronchial circulation; ventilation with room air. *C*, oxygenated blood perfusion of the bronchial circulation; ventilation with 5% O_2 in N_2.

C. Ventilation with 5% O_2 in N_2. The findings in Section 1*B* are supported by experiments in which the bronchial circulation was perfused with oxygenated blood, but ventilation of the lung was carried out with a gas mixture containing 5% O_2 in N_2; the pulmonary circulation was perfused with venous blood as above. In six tests in three experiments reducing the bronchial arterial pressure to zero resulted in a *fall* in both oxygen content and oxygen saturation of the left atrial blood; in one test, in a fourth experiment, no change occurred (Fig. 5*C*). The fall in oxygen saturation of the left atrial blood on reducing the bronchial arterial

pressure to zero is to be expected, because in these experiments the bronchial arterial blood contains a higher oxygen saturation than pulmonary capillary blood exposed to 5% O_2 in N_2.

(2) *Changing the composition of the bronchial arterial blood*

Further evidence that the bronchial circulation joins the pulmonary circulation without traversing the pulmonary capillaries in lungs with a normal flow of blood through the pulmonary circulation was obtained in four isolated right lung preparations ventilated with room air, in which the composition of the blood perfusing the bronchial circulation was changed from oxygenated to venous blood. The results of six observations are shown in Fig. 6, from which it may be seen that substituting venous for oxygenated blood perfusion of the bronchial circulation invariably caused a reduction in oxygen content and saturation of the left atrial blood.

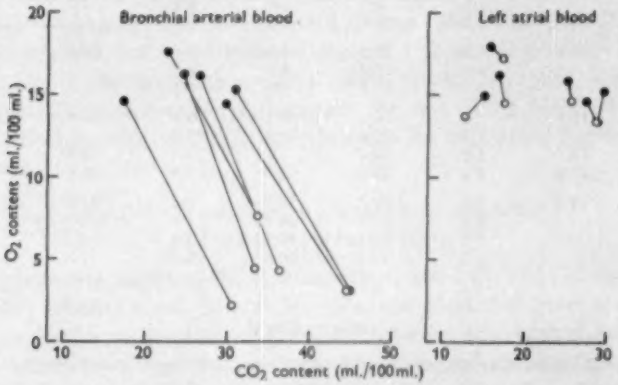

Fig. 6. The effect of changing the composition of the blood perfusing the bronchial circulation from oxygenated (●) to venous (○) blood, shown on the left, on the composition of the left atrial blood, shown on the right.

Metabolism of lung tissue

Information concerning the oxygen consumption of lung tissue *in vivo* was obtained in those experiments in which the bronchopulmonary blood flow in the left diaphragmatic lobe was measured after arrest of ventilation of the lobe for 60–90 min. As described in a previous section of this paper, under these conditions the bronchopulmonary blood is venous in nature owing to the uptake of oxygen by the lung tissue. Oxygen consumption was determined by the Fick principle from the measured bronchopulmonary blood flow and the difference in oxygen content of the blood entering the lung lobe (femoral (bronchial) arterial blood) and the blood

leaving the lobe. The lobar pulmonary vein was ligated, and blood from the cannulated lobar pulmonary artery was used as representative of bronchopulmonary blood. The weight of lung tissue perfused was determined as described in Methods.

The results of fifteen observations in seven experiments are summarized in Table 3. The mean oxygen consumption of lung tissue was 0·48 ml./100 g wet wt./min (range 0·13–0·84 ml./100 g/min).

TABLE 3. Determination of oxygen consumption of the left diaphragmatic lobe *in vivo* by application of the Fick principle. FA, femoral artery; PA, pulmonary artery, representing bronchopulmonary blood

Expt. no.	Dog wt. (kg)	PA blood flow (ml./min)	O_2 content (ml./100 ml.)			Lung tissue wet wt. (g)	O_2 consumption (ml./100 g/min)
			FA	PA	Difference		
2	16·2	2·5	14·8	8·3	6·5	26·4	0·62
3*a*	13·4	2·5	9·3	3·8	5·5	23·9	0·58
b	—	3·3	9·9	3·8	6·1	—	0·84
c	—	2·3	10·6	2·5	8·1	—	0·78
4*a*	16·8	1·9	13·9	5·9	8·0	26·2	0·58
b	—	3·4	12·2	6·2	6·0	—	0·78
c	—	3·5	11·7	6·6	5·1	—	0·68
5*a*	12·3	2·0	21·1	18·1	3·0	28·6	0·21
b	—	1·3	22·0	18·2	3·8	—	0·18
6	12·3	1·2	15·4	7·7	7·7	23·6	0·39
7	13·2	2·4	22·0	12·9	9·1	28·4	0·77
9*a*	19·1	5·6	22·1	19·9	2·2	32·9	0·37
b	—	3·8	22·0	20·8	1·2	—	0·14
c	—	2·8	22·7	20·8	1·9	—	0·16
d	—	2·2	22·4	20·4	2·0	—	0·13

DISCUSSION

The results of our experiments offer the first confirmation by physiological techniques of two important features of the bronchial circulation hitherto based on morphological studies. The first is that the bronchial arterial blood becomes venous in nature before entering the pulmonary circulation, and the second is the identification of the site of the functional anastomoses between the bronchial and pulmonary vascular beds. The interrelationships of these findings and their bearing upon the problem of venous admixture will now be considered in more detail.

Venous nature of bronchopulmonary blood

This was demonstrated by arresting alveolar ventilation of a lobe which was supplied with blood through the bronchial arteries only. The degree of oxygen unsaturation averaged 50 % but varied considerably in different experiments, probably owing to differences in bronchopulmonary blood flow and in lung tissue metabolism. Variations in the bronchial blood flow

during the course of an experiment have been observed previously by Bruner & Schmidt (1947) and by Williams & Towbin (1955).

With regard to oxygen consumption of lung tissue, our value of 0·13–0·84 ml./100 g wet wt./min is lower than that obtained previously by Evans (1912) and Evans & Starling (1913). They found in the heart–lung preparation that the oxygen consumption of lung tissue was equivalent to 1 ml./g *heart* tissue/hr. The corrected value is 1·7–2·1 ml./100 g wet lung tissue/min, assuming the lungs are 80–95% of the weight of the heart (Evans & Starling, 1913). The reason for this discrepancy is not at present clear. It may be the result of the difference in the way the lungs were perfused in the two sets of experiments. On the other hand, it should be mentioned that, in our own experiments, the bronchopulmonary blood may not be representative of either the total bronchial flow through the left diaphragmatic lobe or the oxygen content of venous blood from the lobe, because the fraction of bronchial blood draining the lung lobe via the true bronchial veins could not be measured or analysed. Although it is clear from the present results and those of Berry & I. de B. Daly (1931) that the major part of the bronchial arterial blood drains into the pulmonary circulation, it is not possible to estimate the size of the error introduced in the determination of oxygen consumption by neglecting the bronchial vein blood.

Funcional anastomoses between the bronchial and pulmonary circulations

In experiments made on the left diaphragmatic lobe without its normal pulmonary arterial blood flow, it was demonstrated that there was a free interchange of bronchopulmonary blood between the ligated lobar pulmonary artery and vein and that the blood passed through pulmonary capillaries, although the possibility that some went by way of a network of bronchial capillaries lining respiratory alveoli projecting between the meshes of the muscle in the walls of the respiratory bronchioles cannot be excluded (see Introduction). Under these conditions blood from the bronchial circulation probably flows into the pulmonary circulation via capillary anastomoses between the two circulations which have been demonstrated in morphological studies on the lungs. An alternative pathway by which bronchial blood could enter the pulmonary circulation is through pre-capillary anastomoses between the two circulations. Evidence based on morphological studies suggests that in the lungs of the dog, in contrast to those of man, there are no such anastomoses with the exception of the vasa vasorum of the pulmonary arteries, which arise from the bronchial arteries. Our own experiments, however, do not shed any new light on this problem.

Under normal conditions of blood flow through the pulmonary circulation it is apparent that most of the bronchial blood entering the pulmonary circulation does so, not by way of capillary anastomoses, but by post-capillary communicating vessels which have been demonstrated anatomically by numerous workers (see Introduction). The evidence for this was obtained in the isolated right lung preparation and is presented in sections 1 *B* and *C* and 2 (pp. 613–615). The observed changes in composition of the left atrial blood in response to evoked changes in the bronchial circulation must be due to part, at least, of the bronchial blood entering the pulmonary circulation at a level distal to the pulmonary capillaries. For if the bronchial blood passed solely through alveolar capillaries belonging to the pulmonary and bronchial vascular systems, no change in composition of the left atrial blood would be expected, because the bronchopulmonary blood would be exposed to alveolar air. Estimates based on the quantity of bronchopulmonary blood reaching the left atrium in the isolated right lung preparation indicate that the observed changes in oxygen content of the left atrial blood brought about by either temporary interruption of the bronchial blood flow, or changing the composition of the bronchial arterial blood from oxygenated to venous, can only be explained on the basis that *all* the bronchopulmonary blood reaching the left atrium does so by entering the pulmonary veins directly and without first passing through alveolar capillaries. Owing to the potential sources of error in the methods used, however, the possibility that a small proportion of the bronchial blood reaches the pulmonary circulation through capillary anastomoses cannot be excluded.

It should be mentioned that these conclusions are based on experiments in which the lungs were made hypoxic, either by perfusion of the bronchial circulation with venous blood or by ventilation of the lungs with a gas mixture of low oxygen content. They may be criticized, therefore, on the grounds that the state of the lung blood vessels was abnormal and the normal haemodynamic relationship between the two vascular systems was in some way modified. Indeed, in the absence of hypoxia it was found in the isolated right lung preparation ventilated with air that temporary interruption of the bronchial circulation perfused with oxygenated blood had no consistent effect on the oxygen content and saturation of the left atrial blood. This was confirmed in the entire animal, in which the pressure in the isolated segment of the thoracic aorta which gives rise to the majority of the bronchial arteries was under independent control (unpublished observations). In so far as these two latter types of experiment are concerned, we feel they do not militate against our general conclusions, for as the evidence presented below shows, the expected change in left atrial blood oxygen saturation falls within the experimental error of the analytical

method in our hands and no attempt has yet been made to demonstrate changes in left atrial blood oxygen tension under similar conditions by the microtonometric method. On the other hand, the functional participation of bronchopulmonary post-capillary anastomoses was demonstrated by three different procedures (see sections 1*B* and *C*, and 2, pp. 613–615), and in view of the fact that similar results were obtained under such diverse conditions it seems unlikely that hypoxia was vitiating the results by causing some haemodynamic change common to all three types of experiment.

Relationship to venous admixture

Our finding that most if not all the bronchopulmonary blood enters the pulmonary circulation at a post-capillary level is of some importance with regard to the causes of the alveolar-arterial oxygen tension difference because, being venous in nature, it must contribute to lowering the pO_2 of the blood in the pulmonary veins. The extent to which this occurs depends on two factors, first the relative proportions of the volume flow of bronchial blood into the pulmonary circulation and the total pulmonary blood flow, and secondly the oxygen saturation of the bronchial blood entering the pulmonary circulation, which in turn depends on the metabolic activity of the lung tissue.

With regard to the volume of the bronchopulmonary blood flow, our mean value for the left diaphragmatic lobe is 4·8 ml./min. Using a similar technique, Williams & Towbin (1955) obtained a mean value of 6·5 ml./min. On the basis that this left diaphragmatic lobe constitutes 26·2% of the total lung weight (Rahn & Ross, 1957), the total volume of bronchopulmonary blood reaching the left atrium in our experiments is 18·3 ml./min. If the average cardiac output of our dogs was 2·26 l./min, assuming a value of 0·138 l./min/kg body weight (Marshall, 1926), then the volume of bronchopulmonary blood reaching the left atrium is 0·8% of the cardiac output. Similar calculations from data provided by Williams & Towbin (1955) indicate the value for the whole of the lungs to be 1·4% of the cardiac output. As is shown in Table 4*A*, these values obtained in the entire animal are in agreement with those obtained by other workers. When compared, however, with those obtained in isolated lung preparations perfused simultaneously through the pulmonary and bronchial circulations, they are considerably lower (Table 4*A*, *B*). This apparent discrepancy between the values obtained in the entire animal on the one hand, and in isolated perfused lungs on the other, may be due, at least in part, to the pulmonary blood flow in the latter type of preparation being smaller than normal on a body-weight basis, in spite of the pulmonary perfusion pressure being maintained within the normal range. This explanation is supported by the values for pulmonary blood flow in the right

lung presented in Table 2, and by those obtained by Berry & I. de B. Daly (1931).

Returning now to the contribution of the bronchial circulation to venous admixture: if for the purposes of calculation the value for the bronchopulmonary venous blood flow is taken as 1 % of the cardiac output and the oxygen saturation as 50 %, then taking the left atrial blood as 97 % saturated with oxygen, the end-pulmonary capillary blood would be 0·5 % higher. At this level of oxygen saturation, a difference of 0·5 % corresponds to a pO_2 difference of about 5·5 mm Hg. Although we can make

TABLE 4. Comparison of the values for bronchopulmonary blood flow expressed as a percentage of the pulmonary flow in the entire animal (*A*) and in the isolated lung preparation perfused simultaneously through the pulmonary and bronchial circulations (*B*)

	Authors	Species	Bronchopulmonary blood flow (%)
A.	Bruner & Schmidt (1947)	Dog	0·3–0·83
	Williams & Towbin (1955)	Dog	1·4
	Salisbury, Weil & State (1957)	Dog	0·5–1·0
	Levinson, Cudkowicz, Abelmann & Katznelson (1958)	Man	1·0
	Sapirstein, Sapirstein & Bredemayer (1960)	Rat	3·0*
	Auld, Rudolph & Golinko (1960)	Dog	0·5–0·7
	Present authors	Dog	0·8
B.	Berry & I. de B. Daly (1931)	Dog	2·3, 8·3
	I. de B. Daly (1938)	Guinea-pig	20·0, 13·0
	Present authors	Dog	2·0–10·5

* Bronchial arterial blood flow.

no claim for the ultimate accuracy of this figure in view of the several potential sources of error in the measurements employed for its calculation, it indicates that if the alveolar–arterial oxygen tension difference in the dog is 15–25 mm Hg, then one third to one fifth of this value may be attributed to admixture with bronchopulmonary venous blood.

SUMMARY

1. The distribution of blood between the bronchial and pulmonary vascular systems has been investigated in anaesthetized dogs.

2. Direct measurements of the bronchopulmonary blood flow to the left diaphragmatic lobe were made after ligation of the lobar pulmonary artery and vein. The combined pulmonary artery and vein blood flow ranged from 2·1 to 8·5 ml./min, the oxygen saturation of the blood varying from 91 to 100 %. Changing the ventilating gas mixture of the left diaphragmatic lobe only caused corresponding alterations in the composition of the bronchopulmonary blood, indicating that in the absence of a normal flow of blood through the pulmonary circulation the bronchopulmonary blood traverses the pulmonary capillaries.

3. Cessation of ventilation of the lung lobe for 60–90 min caused the oxygen saturation of the bronchopulmonary blood to fall to a mean value of 50%, which means that the bronchial blood becomes venous on its passage through the lungs.

4. Evidence is presented, using an isolated right lung preparation simultaneously perfused through the pulmonary and bronchial vascular systems, that, when the blood flows normally through the pulmonary circulation, the bronchial blood does not flow through the pulmonary capillaries but enters the pulmonary veins directly, presumably via the bronchopulmonary veins. This bronchopulmonary blood therefore contributes to venous admixture in the pulmonary vein blood.

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